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Maurice Hirst

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THE CHOLINERGIC ACTIONS OF SOME  
ARYLALKYLTRIMETHYLAMMONIUM  
ANALOGUES OF NICOTINE

by

Maurice Hirst, B.Sc., Ph.D.

Department of Pharmacology

Submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy

Faculty of Graduate Studies  
The University of Western Ontario  
London, Canada  
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## ABSTRACT

Synaptic actions of alpha-, beta- and gamma-substituted pyridylalkyltrimethylammonium salts (alkyl chains 1-3 carbons) were studied and then compared to the actions of the analogous phenylalkyltrimethylammonium series. Bioassay methods were chosen such that both "nicotinic" and "muscarinic" characteristics could be evaluated against the stable parasympathomimetic, carbamylcholine. Quantitative information on nicotine-like actions was obtained from the frog rectus abdominis in vitro, the rat phrenic nerve-diaphragm in vitro and some qualitative results were afforded from chick skeletal muscles in vivo. Muscarine-like qualities were obtained from quantitative experiments conducted on the guinea-pig ileum in vitro.

At a later stage, four additional compounds were introduced to allow more insight into structure-activity hypotheses pertinent to the initial studies. These latter drugs comprised isomeric furfuryltrimethylammoniums, 2-tetrahydrofurfuryltrimethylammonium and 1-phenylethyltrimethylammonium and they were evaluated on



the afore-mentioned quantitative preparations.

The two drugs which were most closely related to nicotine, beta-pyridylmethyltrimethylammonium and beta-pyridylethyltrimethylammonium, were again shown to be most active nicotine-like substances, yet the propyl analogue was still extremely potent. This series proved to be the most active on the whole, yet appreciable activity was demonstrated by the members of the gamma- series. The alpha-substituted compounds had low nicotine-like potencies. Comparisons made between the determined potencies of these compounds and the analogous phenyl homologues revealed dissimilarities in the acceptabilities of the variously substituted pyridines and the phenyl system at the nicotinic receptor.

Interpretations of structure-activity inter-relationships were attempted and some intra-atomic distances measured from constructed molecular models of the drugs. Three such distances (4.2 Å, 4.7 Å, 6.8 Å) were noted that were considered of probable significance to nicotinic activity and two of these (4.2 Å, 4.7 Å) coincided with intra-atomic measurements taken from a model of acetylcholine.

Experiments revealed that there was some degree of interchangeability between a furan and pyridine system at the nicotinic receptor. In addition, the reduced furan system was re-examined. These results were all interpreted in a similar manner to that described for

the pyridyl series.

Determinations of muscarine-like potencies showed the pyridyl and phenyl compounds with a one methylene moiety side-chain to be of a similar order of activity to pilocarpine. This level of potency rapidly declined with extension of the side chains above the one carbon group. It was apparent that the above intra-atomic measurements did not lend themselves to analyses of the muscarinic activities and other interpretations were considered after examining the responses afforded by the furan derivatives. At the muscarinic receptor there were much greater similarities between the quantitative responses of the variously substituted pyridylmethyl derivatives and benzyltrimethylammonium than occurred at the nicotinic site. Conversely, there was dissimilarity to the analogous furan compounds.

Synaptic specificity was considered from the above determinations and some selectivity-conferring structural features extracted. The specificity of nicotine was allied to acetyl- $\alpha$ -methylcholine, employing in this consideration the preferentially nicotine-like actions of 1-phenylethyltrimethylammonium.

## I. INTRODUCTION

The materia medica of the pharmacopoeia of the mid-nineteenth century was mostly botanical in content, the more necromantic zoological preparations (for example, the "jus viperinum" of the London Pharmacopoeia of 1745) prominent in the eighteenth century having been deleted. Today the materia medica is largely composed of compounds that originated in chemical laboratories and preparations of plant and animal materials constitute a receding, although still important, minority.

This development can in part be traced to the desire to more fully comprehend the effects that drugs exert on tissues and at the same time upholds the underlying conviction that these effects will eventually be fully rationalised by the natural laws of physics and chemistry. This thesis represents some attempts to advance this sublime quest.

This dissertation has, as its subject matter, correlations between the structures of a group of compounds synthesised by the author and the synaptic activity that they demonstrated on several pharmacological

preparations. To more fully understand this study an historical review is presented to provide background. It includes a survey of the evidence for chemical transmission of nervous impulses, a brief discussion of receptors and receptor theories, and also inspects some foundations on which this 'logical empiricism' of relating chemical structure to physiological activity is based.

## II. HISTORICAL REVIEW

### A. Chemical Transmission Across Synapses

As many authors (Euler, 1951; Dale, 1954; Nachmansohn, 1959; Burn, 1963; McLennan, 1963; Barlow, 1964; Eccles, 1964) have most adequately described the developments that led to the adoption of the chemical transmitter theory, the object of this review will be to gather highlights that are pertinent to this thesis.

#### Early proposals of chemical transmissions

The first proposal for the chemical mediation of neural impulses across synaptic clefts was made by Elliot (1904, 1905). On observing the similarity between applied adrenaline and sympathetic nerve stimulation, he advanced the hypothesis that adrenaline might be the "chemical stimulant liberated on each occasion when the nerve impulse arrives at the periphery". This idea did not find wide acceptance at the time, although Dixon (1906) was sufficiently impressed to advance a like theory embracing the parasympathetic system. However, as no endogenous agent which could mimic the effects of parasympathetic

nerve stimulation was known at that time, this too was repudiated.

Acetylcholine--a possible transmitter

Even so, progress was more rapid with the investigation of parasympathetic synapses and was founded on the properties displayed by acetylcholine.

Hunt had started experimenting with adrenal gland extracts and discovered they possessed "depressor" activity. In one extract that had intense "depressor" effects he found inadequate quantities of choline to account for this action. He subsequently considered that the excessive activity of this extract might be attributed to a labile derivative of choline (Hunt, 1901).

The profound "depressor" activity of acetylcholine was noted by Dale (1914) who detected it in an extract of ergot. He was greatly impressed by the similarity between parasympathetic stimulation and administered acetylcholine, and suggested that this compound might be the transmitter sought by Dixon. Dale also noted that although acetylcholine was a most potent agent, its intense action was of short duration. He inferred that this was consequent on its probable destruction by enzymes in the blood. In addition, Dale introduced the terms "nicotinic" and "muscarinic" to describe the differential actions of acetylcholine: concepts that will receive more attention

later. Basically, as this agent acts on the heart and blood vessels like muscarine (Dixon, 1907) and this action is abolished by atropine, Dale defined this form of activity as muscarine-like. In the presence of atropine sufficiently large doses of acetylcholine resembled the actions of applied nicotine (Langley and Dickinson, 1889) and Dale referred to these as the nicotine-like actions of acetylcholine.

#### A demonstration of chemical transmission

The first direct evidence for chemical transmission of nervous impulses was provided by an inspired experiment performed by Otto Loewi (1921). He wrote of his discovery as follows (Loewi, 1960):

As far back as 1903, I discussed with Walter M. Fletcher from Cambridge, England, then an associate of Marburg, the fact that certain drugs mimic the augmentory as well as the inhibitory effects of the stimulation of sympathetic and/or parasympathetic nerves on their effector organs. During this discussion the idea occurred to me that the terminals of those nerves might contain chemicals, that stimulation might liberate from the nerve terminals and that these chemicals might in turn transmit the nervous impulses to their respective effector organs. At that time I did not see a way to prove the correctness of this hunch, and it entirely slipped my conscious memory until it emerged again in 1920.

The night before Easter Sunday of that year I awoke, turned on the light, and jotted down a few notes on a tiny slip of paper. Then I fell asleep again. It occurred to me at six o'clock in the morning that during the night I had written down something most important, but

I was unable to decipher the scrawl. The next day at three o'clock, the idea returned. It was to the design of an experiment to determine whether or not the hypothesis of chemical transmission that I had uttered seventeen years ago was correct. I got up immediately, went to the laboratory, and performed a simple experiment on a frog heart according to the nocturnal design. I have to describe briefly this experiment since its results became the foundation of the theory of the chemical transmission of the nervous impulse.

The hearts of two frogs were isolated, the first with its nerves the second without. Both hearts were attached to Straub cannulas filled with a little Ringer's solution. The vagus nerve of the first heart was stimulated for a few minutes. Then the Ringer solution that had been in the first heart during the stimulation of the vagus was transferred to the second heart. It slowed and its beats diminished just as if its vagus had been stimulated. Similarly, when the accelerator nerve was stimulated and the Ringer from this period transferred, the second heart speeded up and its beats increased. These results unequivocally proved that the nerves do not influence the heart directly but liberate from their terminals specific chemical substances which, in their turn, cause the well-known modifications of the function of the heart characteristic of the stimulation of its nerves.

Loewi called the substance that was liberated during the period of vagal stimulation "vagusstoff" and the substance responsible for the increased heart rate the "acceleransstoff".

Although Loewi recognised that his "vagusstoff" behaved like an unstable ester of choline, for example, acetylcholine, its identity was not determined for many years.



### Identification of the "vagusstoff"

The establishment of the composition of the heart-slowing substance was beset with problems. The quantities liberated during vagal stimulation were of course inadequate for any form of chemical analysis, and furthermore, it was a labile substance that, in common with acetylcholine, could be rapidly destroyed by extracts of frog heart (Loewi and Navratil, 1926a). A major restriction to considerations of acetylcholine as the neurohumoral agent was that while choline was known to be present in the animal body acetylcholine was not.

Of great value to further progress was the discovery that eserine (physostigmine) potentiated the effects of both "vagusstoff" and acetylcholine despite the presence of the destructive frog heart extracts (Loewi and Navratil, 1926b). This work was expanded by Engelhart and Loewi (1930) and Matthes (1930) who obtained results to support the hypothesis that the destruction of acetylcholine by blood was an enzymatic process, and moreover, they demonstrated that eserine potentiated acetylcholine by specifically preventing its decomposition by the blood-esterase.

In 1929, Dale and Dudley successfully isolated and characterised acetylcholine while examining extracts from the spleen of horse and ox, thereby removing a serious obstacle to the identification of the "vagusstoff".

With the major difficulties solved there was a rapid increase in published results. Chang and Gaddum (1933) determined the quantities of acetylcholine in different tissues and established the founding principles for the bioassay of this substance. On the basis of these principles Dale and Feldberg (1933) and Feldberg and Kraye (1933) finally proved that vagal transmission is mediated by a chemical agent, namely, acetylcholine.

This unequivocal identification was supported by correlative evidence from other laboratories (Dale and Gaddum, 1930; Babkin, Alley and Stavraky, 1932; Feldberg, 1933a, b; Gibbs and Stelozey, 1932; Henderson and Roepke, 1933; Stavraky, 1933).

Chemical transmission at synapses--  
the neuromuscular junction

Riesser and Neuschloss (1921) were the first to show that the application of Ringer's solution, containing acetylcholine, caused a contraction of the isolated frog and toad gastrocnemius muscle. They were impressed by the force of the contractions and, as Dale (1914) before them, by the brevity of its duration. Moreover, they demonstrated that the site of action of acetylcholine appeared to be at the nerve-muscle junction, for applications of acetylcholine to the muscle or to the nerve itself elicited little, if any response.

Unfortunately, it was difficult to extend these findings to mammalian muscle, for simply injecting solutions of acetylcholine rarely produced contractions. Nonetheless, it was soon shown (Frank, Nothmann and Hirsch-Kaufmann, 1922) that when the nerve supply to mammalian voluntary muscles was cut and allowed to degenerate, the denervated muscles became exceedingly sensitive to exogenous acetylcholine. Later, Dale and Gasser (1926) demonstrated that acetylcholine and a number of related quaternary ammonium salts could produce a contraction of the denervated cat gastrocnemius muscle. In addition they showed the similarity between applied acetylcholine and nicotine and showed that the actions of acetylcholine at voluntary muscle were independent of its parasympathetic effects.

Proof for the participation of endogenous acetylcholine as a chemical transmitter at the myoneural synapse was achieved several years later. Dale and his co-workers (Dale and Feldberg, 1934a; Dale, Feldberg and Vogt, 1936) tested various animal preparations for acetylcholine liberated during nerve stimulation. Eserinised fluids were perfused through gastrocnemius muscles of cats, dogs and frogs during and after nerve stimulation, and then bioassayed for acetylcholine. They were able to demonstrate that acetylcholine was regularly contained in effluent collected during the periods of

stimulation and also that acetylcholine was absent during intervals of no stimulation.

Further support for the role of acetylcholine was added by Brown, Dale and Feldberg (1936) who reported that close-arterial injections of acetylcholine could cause a muscle to contract at not less than half the speed of a maximal motor nerve twitch. This response was found to be antagonized by curare, whereas atropine was without effect.

#### Chemical transmission at synapses-- autonomic ganglia

During assays for the quantities of acetylcholine occurring in different tissues, Chang and Gaddum (1933) demonstrated that quite appreciable quantities were to be found in the sympathetic chain, and consequently they reflected that acetylcholine might be a transmitter at sympathetic ganglia. This theory was verified when Kibjadow (1933) stimulated the preganglion nerve of a perfused superior cervical ganglion of the cat and an active substance appeared in the effluent. This experiment was repeated by Feldberg and Gaddum (1934) and Feldberg and Vartiainen (1934) using an eserinated perfusion fluid and they subsequently established the presence of acetylcholine.

#### Chemical transmission at synapses-- adrenal medulla

A further role played by acetylcholine was discovered by Feldberg and Minz (1933). They showed that

acetylcholine was liberated following stimulation of the splanchnic nerve to the adrenal medulla and concurrently that such stimulation released adrenaline from the medulla. On closer scrutiny, Feldberg, Minz and Tsudzimura (1934) firmly established the part played by acetylcholine in the discharge of adrenaline.

#### Chemical transmission--postganglionic sympathetic synapses

There was great confusion in identifying the neurotransmitter at sympathetic synapses. Barger and Dale (1910) suggested it was noradrenaline, yet Loewi's "acceleransstoff" behaved like adrenaline. Additionally, an ingenious, but involved solution to the problem was proposed by Cannon.

Cannon and Uridil (1921) stimulated the splanchnic nerves of a cat, whose heart had been denervated in a previous operation, and the rate increased by a mean of twenty-nine beats per minute. Moreover, after removal of the adrenal glands and repeating the stimulation the heart rate was still above normal by some six beats per minute. Cannon and Uridil concluded from this observation that some substance was being liberated from hepatic sympathetic nerve terminals and further, that this principle was not adrenaline. Similarly, Cannon and Bacq (1931) showed that stimulation of sympathetic fibres that innervate the tail of the cat

released a substance that did not correspond absolutely to the proposed neuro-transmitter, adrenaline. They called this substance "sympathin".

Cannon and Rosenblueth (1933) recognised that there was a duality to sympathetic stimulation, which afforded in some cases a relaxation of decreased activity, and in others a contraction or increased activity. On this basis they suggested that "sympathin" was not one, but two substances. They advanced the theory that initially the released chemical transmitter was homogenous, but interactions with appropriate cell constituents gave rise to two "sympathins", "sympathin" 'E' being formed at excitatory synapses and "sympathin" 'I' being produced at inhibitory synapses. Although this hypothesis was a plausible one it did nothing to clarify the situation.

This confusion encouraged much work to be done on analysing extracts from sympathetic nerves and then, after many hectic years, a light dissipated the darkness. Euler (1946a, b, c) finally demonstrated that the substances present in postganglionic sympathetic nerves accurately resembled noradrenaline and not adrenaline, and he reasoned that the sympathetic nervous system liberated this agent from its terminals. Strong support for this view came from the work of Gaddum and Goodwin (1947) and Peart (1949) who identified noradrenaline as the substance liberated from postganglionic nerves.

Uniformity was established when Holtz, Crednor and Kornberg (1947) and Gaddum, Peart and Vogt (1949) showed that extracts of adrenaline from the adrenal medulla contained a small percentage of noradrenaline. The earlier work of Cannon and his co-workers was therefore explainable, for the adrenal medulla secretes both amines when stimulated, with adrenaline predominating.

#### Classification of synapses and further views

The foregoing text has briefly examined the concept of chemical transmission and reviewed some of the evidence that has gathered. It would be improper to leave it at this point for the subject is far from exhausted. It is generally accepted now that acetylcholine is the transmitter at parasympathetic postganglionic synapses, at ganglia, at the splanchnic synapse to the adrenal medulla, at the nerve-voluntary muscle synaptic clefts and at postganglionic synapses to sweat glands (Dale and Feldberg, 1934b) and certain blood vessels (Euler and Gaddum, 1931). The latter synapses are anatomically a part of the sympathetic system and this ambiguity led Dale (1933) to advocate the following terminology. He suggested that the confusing situation presented by the classical anatomical differentiation of the autonomic nervous system into parasympathetic and sympathetic divisions might be physiologically clarified,

by calling the nerve fibres that release "adrenaline-like" substances--"adrenergic" fibres and the nerve fibres that release acetylcholine--"cholinergic" fibres. The peripheral nervous system may therefore be divided more practically into cholinergic and adrenergic fibres, depending inherently on the nature of the transmitter released. The nerves that are considered to be cholinergic have been alluded to above and adrenergic nerves include all the postganglionic fibres of the sympathetic system, bar those classified as cholinergic.

The chemical mediation of neural impulses finds wide acceptance now, but the status is far from being a static one. Although not refuting the roles played by acetylcholine and noradrenaline, evidence has been accumulating that underlines the complexities of chemical transmission (Volle, 1963; Zaimis, 1964; Trendelenberg, 1965; Hamilton and Rubinstein, 1968). For instance, atropine, which is classically considered as an antagonist to acetylcholine at the postganglionic parasympathetic end site has been shown to be capable of interfering with ganglionic transmission (Fink and Cervoni, 1953; Bainbridge and Brown, 1960; Trendelenberg, 1961a; Takeshige and Volle, 1964a, b, c). Perhaps related to this has been the discovery that there is apparently a "muscarinic" component to ganglionic enervation which permits postganglionic stimulation to



occur in the presence of "nicotinic" blocking drugs (Rubinstein, 1966; Hamilton and Rubinstein, 1968). In addition, Burn and Rand (1959, 1960, 1962, 1965) have suggested that the release of noradrenaline from the adrenergic fibre is not a direct process following upon the arrival of an impulse. They claim instead that the nerve impulse in an adrenergic neuron first liberates acetylcholine and that this acetylcholine, acting within the same fibre, then liberates noradrenaline.

Although this view of an intraneuronal adrenal medulla has not been unanimously acclaimed, it serves to whet the continuing interest in synaptic transmission.

#### B. The Existence and Interactions of "Receptors"

The hypothesis that interactions of drugs with special defined areas on effector cells produces biological responses was first presented by Langley (1878, 1907) and this theory formed the essential basis for Ehrlich's investigations on chemotherapy. Ehrlich viewed a receptor as being "that combining group of the protoplasmic molecule to which a foreign group, which introduced, attaches itself" (Ehrlich and Morgenroth, 1910). He deduced also that a receptor is materially small, being only a minute portion of the cell. This point was strongly supported by the sophisticated quantitative researches of Clark (1933, 1937) who

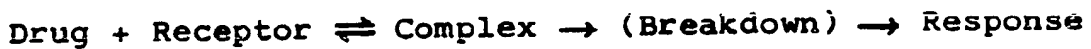
demonstrated that the number of molecules of acetylcholine that produced physiological responses could not conceivably cover more than a very small fraction of the cell surface. Similar calculations revealed that drugs fall into two classes, namely, those which exert an effect at "receptors" and others that only produce effects when given in amounts which would suffice to form a monomolecular layer over the whole area of the cellular surface. This latter type of drug-cell interaction may produce the biological response by physically or physiochemically interfering with normal cell function. This could therefore be correlated to the Meyer-Overton hypothesis which proposes that anaesthetic potential is related to a high oil-water partition coefficient.

The calculations that supported the former type of drug-cell interaction provided strong evidence for the existence of active sites or receptors and it was presumed that drugs act by complexing with these.

#### The occupation theories of drug-receptor interactions

If the biological response is plotted against the logarithm of the effective dose of a stimulating, or agonistic, drug an approximately sigmoid curve is described. Although the precise significance of this

curve is in doubt its close resemblance to the Langemuir adsorption isotherm prompted Clark (1937) to reflect that adsorption and drug action might be fundamentally similar processes, whereby the response to an agonist might be considered as a consequence of its adsorption at a receptor. This is expressible in the form;



Clark's theory assumed that the response is proportional to the number of agonist-receptor interactions and furthermore, that full occupancy of the receptors is essential for maximum response. In addition, it was implied, on an all or none basis, that the rate of formation was the most important step for the production of the response.

Recently, Ariens and his co-workers (Ariens, 1954; Ariens, Simonis and de Groot, 1955; Ariens, Van Rossum and Simonis, 1956) and independently, Stephenson (1956), have proposed an extension to the basic Clark hypothesis. They suggest that the activity of a drug depends not only on its adsorbability (affinity), but on another property termed "intrinsic activity" (by Ariens), or "efficacy" (by Stephenson), and this factor is a measure of the complex-stimulating ability of the drug. For example, there are substances which are intermediate in response-producing activity that are

called "dualists" (by Ariens) or, more appropriately, "partial agonists" (by Stephenson). These substances have the following properties. They are themselves incapable of producing the maximum response of which the tissue is capable, whatever concentration is used; impede the response to more efficacious compounds and do not act additively (presumably because they occupy receptors that could more profitably complex with the more active agent). Ariens (1954) regards antagonists (compounds that block the normal response to an agonist) as having an "intrinsic activity" of zero, agonists that produce maximum tissue responses as having "intrinsic activities" of unity and the "dualists" as having "intrinsic activities" between these extremes. The biological effect is therefore regarded as being dependent on the "intrinsic activity" and on the proportion of receptors occupied. A maximum response being produced when a drug of "intrinsic activity" of unity complexes with all available receptors.

Stephenson (1956) uses a similar, yet modified, approach. In his view, a biological stimulus (which is assumed to be related to the biological response) is afforded by the interaction of a drug with an "efficacy" which can have any positive value from zero upwards, with a proportion of the available receptors. An important difference therefore is that employing the

latter hypothesis, a maximum biological stimulus may be produced by a potent agonist in the absence of total receptor occupancy, and consequently Stephenson postulated that "spare" receptors may exist. This concept was supported by the work of Nickerson (1956) who, using low concentrations of an irreversible antagonist, demonstrated that such a potent agonist could still elicit maximal responses. Ariens, Van Rossum and Koopman (1960) also investigated the problem of reserve receptors and were unable to refute their presence. Consequently they allied their results to the "intrinsic activity" hypothesis.

Whether supernumerary receptors exist or not both Ariens' and Stephenson's hypotheses equate response (or stimulus) to a relatively static state of receptor occupation.

### The "rate" theory of drug action

Clark's theory of drug action was also extensively remodelled by other groups (Paton, 1961; Paton and Rang, 1966; Gill, 1965) who emphasised that excitation by a stimulant drug may be proportional to the rate of formation of the drug-receptor complex, rather than the proportion of receptors occupied by the drug. Hence, the properties of a drug would be definable by two rate constants; the rate of association

with the receptor and the rate of dissociation from the receptor. The affinity of the drug would then be related to the equilibrium situation, where an optimal density of transmutable drug-receptor combinations exists. This theory offers a model for the otherwise mystical property of "intrinsic activity".

Unlike the "occupation" theory which would predict that the response should rise exponentially to a maximum level as receptors became occupied, the "rate" theory predicts that the response should be maximal at the onset of exposure, when the total complement of receptors are available for occupancy, and the response would then fall to the equilibrium plateau.

Removal of the drug should, by the "rate" theory cause an immediate cessation of the response (if diffusion of the substance is rapid compared to the rate of association), whereas the "occupation" theories require the effect of the drug to decrease as the drug-receptor complexes dissociate.

Ariens and Simonis (1967) have attempted to differentiate the theories experimentally by exposing various drugs to selected isolated tissues and measured changes in membrane potential. They conclude that the observed effects do not suggest that the rate of receptor occupation is a critical factor in the cases studied. However, they do have reservations on

wholeheartedly supporting the occupation theories, for they conclude that rates of tissue penetration by the drugs may be limiting parameters which mask the true situation and prevent a critical appraisal (cf., Waud, 1968).

It is not a simple matter therefore to choose between the theories, for each has its merits and its obliquities, each may be attacked and defended. Both theories do however permit relative measurements to be made of drugs that allow rational categorisation. Indeed, incontestable differences between the theories are not major issues when compared to the more precarious receptor problems. For discussions on the molecular constitutions of receptors and reflections on how the primary drug-receptor interactions may give rise to the tissue response the reader is directed to the following sources of reference (Chagas, Penna Franca, Nishie and Garcia, 1958; Chagas, 1959; Ehrenpreis, 1960; Ehrenpreis and Fishman, 1960; Ehrenpreis and Kellock, 1960; Cavallito, 1962; Belleau, 1964; Belleau and Lacasse, 1964; Ehrenpreis, 1967).

### C. Structure and Activity

At the end of the nineteenth century, as the major chemical and pharmaceutical industries began to develop, a need arose for methods to assess the

quantitative chemotherapeutic activities of scores of new compounds. Paul Ehrlich may be credited with one of the earliest attempts at systematic analysis of drug potency with his concepts of "dosis toxica" and "dosis tolerata", but clearly, more was required. The development of the principles and some of the early methods of bioassay may be traced to Clark (1937), and this work was instrumental in establishing chemical pharmacology. The adoption of the bioassay (which has been defined by Bliss (1952) as "the determination of the potency of the physical, chemical or biological agent by means of a biological indicator") has led to the national and international standardisation of several biological therapeutic agents, including digitalis extracts and insulin preparations, and additionally enabled the potencies of new drugs to be compared to established ones. This in turn, as more and more substances were tested, generated curiosity into what constituted the molecular-structural requirements essential for biological activity. As marked changes in biological activity frequently accompanied slight changes in chemical structure, many authors (for example, Gill, 1965) speculated on the concept of "lock and key" relationships (cf., Fischer, 1894) applying to drug-receptor interactions that is, where the receptor has a spatially complementary structure to the drug which enables optimal binding



between the two to occur. As this thesis reports on cholinergic activities this structure-activity discussion will concentrate on the interactions of several drugs with the cholinergic receptors.

Structure-activity relationships--acetylcholine analogues and muscarinic activity

The historical developments that led to the acceptance of acetylcholine as a neuro-transmitter have been noted. This fascicule will survey the synthetic agents whose activities aid an understanding of cholinergic requirements at the molecular level. It must be emphasised that the activities of acetylcholine analogues are often as diversified as the actions of acetylcholine itself, and consequently, although this section concentrates on the muscarinic actions, it must not be assumed that the compounds discussed possess unadulterated postsynaptic stimulating ability.

Acetylcholine may be regarded as a derivative of the simple tetramethylammonium ion which itself possesses a feeble muscarinic activity (Burn and Dale, 1915; Clark and Raventos, 1937). In attempts by many investigators to deduce, from structure-activity relationships, a working hypothesis for its stimulant action the neuro-transmitter molecule has been extensively adapted. Analogues have been prepared that examine the effects

of changing the N-alkyl substitution (Stehle, Melville and Oldham, 1936; Holton and Ing, 1949; Barlow, Scott and Stephenson, 1963) and other substances have been prepared wherein the nitrogen atom is replaced by phosphorus and arsenic (Welch and Roepke, 1935) and by tertiary sulphur (Ing, Kordik and Tudor Williams, 1952). The results obtained from these investigations indicate that activity is exceedingly dependent on the size of the onium (positively charged) group. Increasing the size of the central atom--nitrogen, phosphorus, sulphur, arsenic causes a decrease in effectiveness. Holton and Ing (1949) suggested that this decline is related to the increasing spread of the attached methyl groups (the distance is approximately 27% to 35% greater in the phosphorus and arsenic analogues than in acetylcholine), but it is equally probable that the controlling factor is the decreasing energy of bonding between the cation and the putative, complementary negative charge of the receptor. Gradual replacement of the N-methyl groups by N-ethyls similarly reduces the activity, the triethyl analogue being inactive (Stehle, Melville and Oldham, 1936; Holton and Ing, 1949), and Ing (1949) argued that the manner in which this decrease was effected presupposed that the onium group is adsorbed at a planar surface or hemi-spherical cavity in the receptor. This assumption adequately explains the experimental details

and although more sophisticated activity parameters have since been measured (Scott, 1962; Barlow, Scott and Stephenson, 1963) the final analysis supports the inclusion of at least two methyl groups for moderate efficacy (Barlow, 1964).

Other modifications to the acetylcholine molecule, changing the acyl (acetyl) group (Abderhalden, Paffrath and Sickel, 1925; Wertheimer and Paffrath, 1925; Chang and Gaddum, 1933; Le Heux, 1921; Simonart, 1932; Wurzel, 1959), or altering the number of methylenes of the choline moiety (Hunt and Taveau, 1911; Hunt and Renshaw, 1925, 1934) invariably causes a reduction of muscarine-like activity. However, one of these analogues, carbamylcholine, a relatively stable ester of choline (Kreitmair, 1932; Ammon, 1935; Roepke, 1937), has been used therapeutically to manage urinary retention and states of gastric atony, but its nicotinic side-effects have seriously limited this usage. Its resistance to enzymatic and pH induced hydrolysis, combined with its relatively high muscarinic and nicotinic activities made it the comparative drug of choice in the studies described in this thesis.

#### Structure-activity relationships-- muscarine and related compounds

Although the ability of muscarine to mimic the

effects of parasympathetic stimulation was one of the founding certitudes on autonomic pharmacology (Schmiedeberg and Koppe, 1869) the elucidation of its structure is a comparatively recent achievement (Eugster, 1956; Eugster and Waser, 1954, 1957; Kogl, Salemnink and Schuller, 1960; Hardegger and Lohse, 1957). It may be regarded structurally as a cyclic ether derivative of acetylcholine, a fact that presumably accounts for its high activity. It is worthy of note that the molecule has three asymmetric (optically active) carbon atoms and stereochemical alterations to these centres results in drastic activity changes (Waser, 1958, 1962; Van Rossum, 1960; Gyermek and Unna, 1958) indicating the frequently encountered importance of stereochemistry to drug-receptor interactions. Other structural analogues of muscarine include the highly active 1,3-dioxolanes studied by Fourneau, Bovet, Bovet and Montezin (1944). These compounds, F 2268 and F 2581, may also be viewed as cyclic derivatives of acetylcholine. Unlike the majority of the other comparative determinations estimates of the activity of F 2268 vary. Carr and Riggs (1951) found that acetylcholine, acetyl-~~f~~-methylcholine and F 2268 were almost equiactive at lowering the cat blood pressure, whereas Salle and Valade (1954) reported that F 2268 was only one hundredth to one fiftieth as active as acetylcholine on the dog

blood pressure and the intestine and bronchial muscle of the guinea pig. These results may reflect differences in cholinesterase content of the tissues studied and the stability of the drugs to the enzyme, but stereochemical factors may also be involved (Triggle and Belleau, 1962).

Muscarinic activity was also found in 2-furfuryltrimethylammonium (Furmethide) by Fellows and Livingston (1940), and this substance bears an obvious structural resemblance to muscarine. It will be noted that if the molecule possessed a methyl group adjacent to the oxygen (becoming 5-methylfurmethide) it would resemble muscarine more closely. Ing (1949) from a study of the molecular structures of compounds with muscarinic activity, theorised that the length of the chain attached to the onium group was of great importance and was optimal when it was comparable with five linear atoms (other than hydrogen). To test this theory he synthesized 5-methylfurmethide, and indeed it proved to be considerably more active than furmethide itself (Ing, Kordik and Tudor Williams, 1952). Although this 'five atom rule' can be applied to many molecules the existence of some profound exceptions (e.g., Jacob, Marszak, Bardisa, Marszak-Fleury and Epsztein, 1952) makes it apparent that the 'rule' constitutes an oversimplification of muscarinic requirements.

Structure-activity relationships--  
muscarinic specificity

Generally the cyclic analogues of acetylcholine are quite specifically muscarinic in character, that is, they tend to be much less active at other non-muscarinic cholinergic synapses. These substances are conformationally more restricted than acetylcholine, as the ring system provides "backbone" to the molecules and perhaps this is related to specificity. Recently, Martin-Smith, Smail and Stenlake (1967) have examined the evidence for the participation of different conformations (spacial arrangements) of acetylcholine at nicotinic and muscarinic synapses. They note that X-ray diffraction studies of crystalline acetylcholine bromide have shown the adoption of a quasi-ring structure wherein the positively charged nitrogen atom is circumjacent to the ether oxygen (Canepa, Pauling and Sorum, 1966), and this picture of the molecular geometry is very similar to that found for L(+)-muscarine iodide (Jellinek, 1957). This structural information, although pleasing, refers exclusively to the rather static state of crystallinity and it becomes less didactic when the physical state is altered. Acetylcholine exerts its effects as molecules in a liquid medium, that is as a solution. In this state the movement of the molecules is much less restricted and low-energy conformations,

where there is a minimum of atomic interactions, are preferentially adopted. However, the situation is one of constant flux and higher energy conformations do exist as short-lived 'species'. Gill (1965) has calculated, from considerations of steric requirements and energies of interaction, that the quasi-ring, muscarine-like conformer of acetylcholine is likely to be present in solution to the extent of four percent. In terms of concentration this is not excessive, yet, as Gill points out, there is no evidence to indicate that the receptor shows a preference for the most energetically favourable form of the molecule. Hence, the above-mentioned conformer of acetylcholine could well be acting at the post-synaptic sites.

It has been mentioned above that extending or reducing the length of the choline portion of acetylcholine reduces muscarinic activity. However, altering the choline moiety by substituting a methyl group for a methylene proton curiously confers specificity on the sites of action of the modified acetylcholines.

Acetyl- $\alpha$ -methylcholine, has nicotinic activity but little muscarinic activity and is discussed later. Acetyl-

-methylcholine possesses diametrically opposite actions, having muscarine-like activity but feeble nicotine-like activity (Simonart, 1932; Wurzel, 1959). Introducing a methyl group in this manner creates an asymmetric centre (optically active centre) and Major and Cline (1935)

resolved the racemic compound and found that the (+) -isomer is much more active than the (-) -isomer. Ellenbroek and Van Rossum (1960) and independently Beckett, Clitherow and Harper (1960) have shown that the more active isomer, (+)-acetyl- ~~$\beta$~~ -methylcholine, has the same stereochemical configuration as natural muscarine. Although stereochemical consistency is therefore apparent, it is worthy of note that the introduction of the branched methyl group would increase the non-bonding interactions and energy of the 'muscarinic' conformation of acetylcholine discussed above.

Cavallito and Gray (1960), in a review of the pharmacological action of quaternary ammonium salts, present a more general theory. They call attention to the influence of small structural changes on muscarinic potency and suggest that muscarinic activity is favoured when substituents are placed on the beta-carbon atom from the cationic head affording a protuberant methylene-trimethylammonium neck. This generality successfully encompasses the preferential post-ganglionic postsynaptic activities of muscarine, acetyl- ~~$\beta$~~ -methylcholine, furmethide, 5-methyl furmethide and many other compounds, yet alone it is not exclusive, for other substances such as the muscarones are equivalently beta-substituted and are also potent nicotine-like agents (Gyermek and Unna, 1960; Waser, 1962).



Structure-activity relationships--acetylcholine analogues and nicotinic activity

It has been mentioned previously that tetramethylammonium, the basic cationic head of acetylcholine, is only feebly active at the postganglionic postsynaptic site, with approximately one thousandth the activity of the neuro-transmitter (Clark and Raventos, 1937). In contrast, it is about one hundredth as active at the neuro-muscular junction (Clark and Raventos, 1937) and surprisingly, it is more active than acetylcholine in raising the blood pressure of the atropinised, anaesthetised cat (Burn and Dale, 1915). It may be argued that this reflects the rapid destruction of acetylcholine "in vivo", and although the importance of this factor cannot be denied, the tetramethylammonium compound was still comparable in activity with nicotine.

The fidelity with which this uncomplicated cation reproduced the nicotine-like actions of acetylcholine prompted many workers to investigate its homologues. It was demonstrated that the lower members of the series were agonists and that larger increases in chain length (above five methylenes) produced partial agonists and then antagonists (Raventos, 1937; Ariens, Simonis and de Groot, 1954). The pentyltrimethylammonium compound, the homologue most closely resembling acetylcholine,

proved to be exceedingly active both at ganglia (Alles and Knoeffel, 1939, quoted by Bovet and Bovet-Nitti, 1948; Willey, 1955) and the neuro-muscular synapse (Ing, Kordik and Tudor Williams, 1952; Ariens, Simonis and de Groot, 1954; Willey, 1955; Philipott and Schlag, 1956).

In general, all the modifications to the acetylcholine molecule mentioned in the previous section, namely, replacement of the nitrogen atom, alterations to the cationic head and varying the choline portion resulted in decreased activity, and the reader is directed to the quoted references for more details.

Altering the acyl group affects the ability to elevate the blood pressure, in the presence of atropine, in much the same way as it affects the ability to cause contracture of slow muscle fibres. Several of these analogues are apparently more active than acetylcholine, but the results are rendered difficult to interpret, especially in the determinations of ganglion stimulating ability, by the indeterminate roles being played by the pseudocholinesterases. (Ormerod (1956) has demonstrated an improved method of estimating ganglionic activity in which the drug is applied to the perfused ganglion in the presence of eserine, however, the majority of classical nicotinic agents have yet to be estimated by this method.) Nicotine-like activity does

not appear to be as structurally restricted as muscarine-like activity, for many of the analogues that possess intense ganglion stimulant activity have large aliphatic or aromatic esters, for example, valerylcholine, benzoylcholine, and phenacetylcholine are all more active than acetylcholine (Hey, 1952; Willey, 1955; Sekul and Holland, 1961). Greater selectiveness is however demonstrated at the neuro-muscular junction, for although relatively large acyl (aliphatic ester) groups are tolerated (even ones that are terminally substituted by aromatic moieties), aryl (aromatic acid) esters are feeble agonists (Willey, 1955; Ormerod, 1956; Erspamer and Glasser, 1958).

Aliphatic ethers of choline are relatively inactive at stimulating ganglia or at causing contractures of slow muscle fibres (Dale, 1914; Simonart, 1932, 1934; Ing, Kordik and Tudor Williams, 1952). Aromatic ethers of choline are, on the other hand, exceedingly active in elevating the blood pressure (Hey, 1952), yet their ability to elicit a twitch of the cat gastrocnemius (Willey, 1955) indicates that their depolarising activity at the neuro-muscular junction is much less pronounced. Hey (1949) had suggested that maximum nicotine-like stimulant action was to be found in ions of the choline phenyl ether type where the oxygen atom was subjected to a maximum mesomeric electron 'withdrawal' by the

aromatic ring. This 'withdrawal' of electrons results in a partial positive charge residing on the oxygen atom, the equivalent negative charge (from 'appropriated' elections) serving to increase the electropositivity of the aromatic system. Hey investigated a number of substituted phenyl-choline ethers to determine whether a correlation between the electron-withdrawing, or electron-releasing, properties of the substituents and ganglion stimulating activity existed and discovered that halogen substituents at the meta- positions afforded much increased activity, whereas methyl substituents at these positions resulted in decreased activity (Hey, 1952; Ambache and Robertson, 1953). It is interesting that if the methyl groups are 2,6-substituted, the resultant compound (choline-2,6-xylyl ether) is devoid of ganglion stimulating ability (Hey, 1952; Clark and Jana, 1966). Although aliphatic ethers are relatively inactive as stated above, aliphatic ketones can display intense activity (Ing, Kordik and Tudor Williams, 1952; Willey, 1955). As it is permissible to ascribe a partial positive charge to the carbon of a carbonyl group, this activity is readily accommodated by Hey's theory. Similarly, the nicotinic activities associated with the aforementioned muscarones might be allied to the polarisation of their carbonyl groups.

Polarisation, as well as providing partial

positive charges produces equivalent partial negative charges which are generally in close proximity to one another. In consequence, Sekul and Holland (1961a, b) from a study of the effect of some esters of choline on the blood pressure of anaesthetised dogs suggested that the partial negative charge was the one of importance. It is difficult to conceive of an experiment that could determine the relative importance of these opposing hypotheses, although Barlow (1964) poses some acute objections to the partial negative charge theory. More recently an extended series of choline-phenyl ether analogues have been investigated for their relative nicotinic activities (Coleman, Hume and Holland, 1965) and the results obtained suggest that there may be an optimum electron density requirement, after which a further decrease in the basicity of the ether oxygen leads to reduced potency.

It is unfortunate that this may be equally well interpreted as either having a partial positive charge more positive, or a partial negative charge become less negative for the ground state of the molecular charge is unknown. Clark, Daves and Williams (1968) have studied some restricted phenylcholines and have verified that nicotine-like activity in choline-phenyl ethers is greatly dependent on the choline portion lying in the same plane as the phenyl ring. In this situation the  $\pi$ -electrons

of the aromatic ring and the oxygen non-bonded electrons can resonate maximally, thereby reducing the basic character of the oxygen atom. The occurrence of this resonance is supported by the nuclear magnetic resonance studies of Brouwer, Mackor and MacLean (1966) who demonstrated that ring protonation occurs in 2,3- and 2,5-dimethylanisole, whereas the ether oxygen is protonated exclusively in 2,6-dimethylanisole.

The 2,6-dimethyl substitution must sterically interfere with the free rotation of the alkyl ether moiety preventing such a resonance. This can obviously relate to the non stimulating ability of choline-2,6-xylyl ether, where steric restrictions would be similarly present, and the molecule would be critically unable to adopt the required planar conformation.

#### Structure-activity relationships--the phenylalkyltrialkylammonium salts

The phenylalkylamines may be regarded as either derivatives of tetramethylammonium, or analogues of the abovementioned phenylcholine ethers in which the ether oxygen has been replaced by a chain methylene. The quaternary salts of these interesting compounds possess activities that support many of the observations and structure-activity hypotheses alluded to in this dissertation.

The first members of the homologous series were investigated by Hunt (1926). He found that both phenyltrimethylammonium and benzyltrimethylammonium in low doses caused a fall in blood pressure in the anaesthetised cat and this depression was prevented by atropine. Larger doses of these drugs produced an atropine reversible cardiac slowing. In atropine pre-treated spinal cats the drugs raised the blood pressure and accelerated the heart an action blocked by large doses of nicotine. Hunt concluded that both compounds were able to mimic the multiple effects of acetylcholine and although no comparative quantitative experiments were performed he regarded benzyltrimethylammonium as having profound muscarine-like activity.

Ing and Wright (1931, 1933) studied the effects of phenyltrimethylammonium, benzyltrimethylammonium and phenylethyltrimethylammonium at the neuro-muscular junction and noted that all of the compounds were active and produced contractures like tetramethylammonium. Phenylethyltrimethylammonium was also examined by Hunt and Renshaw (1933) who demonstrated that it caused a fall and then a rise of the blood pressure of the anaesthetised cat. The depression was prevented by atropine and this was deemed a muscarinic action. Comparatively it was a similar depressant action to that produced by phenyltrimethylammonium, but it was much shallower than benzyltrimethylammonium.

On pre-treatment with atropine, phenylethyltrimethylammonium only elevated the blood pressure and this was prevented by very high doses of nicotine. Furthermore, additional experiments revealed that on a weight basis phenylethyltrimethylammonium appeared to be equiactive with nicotine and much more active than tetramethylammonium. This was supported by the independent work of Alles (1933) who reported that phenylethyltrimethylammonium produced a somewhat greater pressor response than an equimolar concentration of nicotine in the anaesthetised dog.

The first quantitative studies were performed by Raventos (1937), who tested the compounds for their ability to produce a contracture of the isolated rat intestine. He noted that on an equiactive molar concentration scale phenyltrimethylammonium and phenylethyltrimethylammonium were as active as tetramethylammonium, whereas benzyltrimethylammonium was ten times more active. From this and other observations he suggested that benzyltrimethylammonium was comparable in muscarine-like activity to n-butyltrimethylammonium and thus he supported an earlier conclusion of Hunt (1926). He reasoned therefore that a phenyl group substituent on tetramethylammonium behaved more like the addition of three straight chain methylenes rather than a six carbon atom moiety, denoting that changes in activity were not necessarily based on the sum total of carbon atoms in the



side chain of tetramethylammonium. Alles in 1944 (according to Lee and Shideman, 1959) confirmed Raventos' conclusions by showing the marked similarities existing between phenylalkyltrimethylammonium compounds and analogous aliphatic salts of the type  $\text{CH}_3(\text{CH}_2)_{3+n} \text{N}^+(\text{CH}_3)_3$ .

The synaptic properties of the phenylalkyltrialkylammonium salts demonstrated by these earlier workers have been supported by more recent experiments of Lee and Shideman (1959) and Wong and Long (1962). Generally the compounds possess comparable nicotine-like activities to the aliphatic homologues of tetramethylammonium and phenylcholine ethers and the muscarinic actions of benzyltrimethylammonium serve as an additional example for the "methylene neck" theory of Cavallito and Gray (1960) (cf., Wong and Long, 1962).

Additionally, this group of compounds will, as described later, be involved in a structural correlation discussion that attempts to relate nicotine and, by way of intermediate analogues, acetylcholine itself.

#### Structure-activity relationships-- nicotine and related compounds

Although it is recognised that nicotine appears

peripherally to be capable of producing direct responses in the absence of identifiable ganglia (Evans and Schild, 1953a, b; Middleton, Obert, Prager and Middleton, 1956; Lee and Shideman, 1959) and of indirectly causing sympathomimetic responses (Coon and Rothman, 1940; Evans and Schild, 1953a, b; Lee, McCarthy, Zodrow and Shideman, 1960) the effects at the ganglion cell and the neuro-muscular junction, the more classical interpretations of nicotinic activity, are employed in this investigation of structure and action.

Nicotine is a major alkaloid ceded by *Nicotiana* species and chemically is a substituted pyrrolidine, namely 1-methyl-2-(3-pyridyl)-pyrrolidine. Its chemistry and pharmacology have recently been the subjects of review by Pailer (1965) and Trendelenburg (1965) and this discussion will attempt to combine the two by examining the structural features that are required for synaptic stimulation.

It is somewhat more difficult to establish recognisable similarities between acetylcholine and nicotine than between acetylcholine and muscarine and the majority of compounds discussed up to this point. Initially, there appears to be little in the nicotine molecule to serve as a focus about which to drape additional atoms common to this molecule and to acetylated choline, yet such a focus exists below the

physicochemical surface.

Nicotine is a base that contains two tertiary nitrogen atoms, the pyrrolidine nitrogen is strongly basic, the pyridine nitrogen is weakly basic. It is soluble in low polarity solvents and moderately soluble in water. Most of the compounds previously discussed are positively charged quaternary salts and with few exceptions, are exceedingly soluble in aqueous media. Taylor (1951) considered the solubility of nicotine in water and deduced, from dissociation constants determined by Vickery and Pucher (1929), that at physiological pH nicotine is largely present as the univalent (pyrrolidinium) cationic form. He reflected that the pharmacological activity of nicotine might be dependent on this protonated nicotinium ion.

Quaternary nicotine-metho derivatives were prepared by Barlow and Dobson (1954) and subjected to pharmacological examinations (Burn, quoted by Barlow and Dobson, 1954; Gillis and Lewis, 1955). The results obtained suggested that nicotine monomethiodide was somewhat more potent than nicotine at stimulating skeletal muscle but less active at autonomic ganglia (cf., Barlow and Hamilton, 1962a). The isomeric nicotine isomethiodide was found to be inactive at stimulating the frog rectus abdominis muscle. Taylor's hypothesis was strongly supported by this evidence, for although slight

differences were obviously present quaternisation of the N-methylpyrrolidine moiety did not greatly modify synaptic activity.

More direct methods of assessing the importance of protonation to the peripheral pharmacological actions of nicotine have been attempted with moderate success (Barlow and Hamilton, 1962b; Hamilton, 1963; Burgen, 1965). In these, bioassays were performed in which the muscle-bathing solutions were subjected to pH variation on either side of physiological, with the results correlated to nicotinium ion concentrations. Although attention was drawn to the inherent possibility of altering the receptor environment by these non-physiological pH excesses the observations made supported the importance of the nicotinium ion. Indirect evidence is available also. Metabolites of nicotine and related, co-existing tobacco alkaloids which bear a strong structural resemblance to nicotine, yet in which the basicity of the pyrrolidine has been markedly reduced, are much less potent "nicotinic" agents (Kitamura, 1958; Bowman, Kennedy, Wada and McKennis, 1959; Clark, Rand and Vanov, 1965). It would therefore appear that the nicotinium cation plays an important role. Acceptance of this view permits the first structural correlation between nicotine and acetylcholine to be drawn, for both molecules are appreciably or wholly ionic at physiological pH, and may

therefore be regarded as complexing with a common anionic receptor site.

It would be a gross oversimplification to assume that nicotine functions solely as a quaternised pyrrolidinium ion, for bioassays of such compounds have revealed that the most potent, N,N-dimethylpyrrolidine, is less active than tetramethylammonium (Barlow, Scott and Stephenson, 1967) and consequently the pyridine ring system must be implicated in receptor complexing.

Not only does the pyridine ring appear to be of importance to the activity of nicotine, but the position of substitution on this ring somewhat influences this activity. Several workers (Macht and Davis, 1934; Oosterhuis and Wibaut, 1936; Craig, 1934; Heymans and Bouckaert, 1941) have shown that derivatives in which the pyridine nucleus is substituted in the alpha- position are much less active than the corresponding derivatives in the beta- series. The alpha-nicotine is less toxic than nicotine and alpha-nornicotine has only one half the toxicity of naturally occurring nornicotine.

The effects of replacing the pyridine nucleus by different aliphatic and aromatic groups have been studied. In 1928, La Forge synthesised 2-methyl- and 2-phenylpyrrolidine and their N-methyl derivatives, and found that they were far less toxic than nicotine. Similarly, Craig and Hixon (1931) and Craig (1933)

examined the variation in toxicity with different substituents in an effort to establish the 'toxifore' grouping and concluded that toxicity is associated with electronegative 2-position substituents. It should be noted that the assays used in the aforementioned researches employed death as the titre. The results obtained are therefore not necessarily compatible with those afforded by more sophisticated whole animal or isolated organ preparations used to assess synaptic potency, and it is most unfortunate that more acceptable bioassays were not performed. Nonetheless, although the results are pharmacologically restricted the importance of an adequately substituted pyridyl nucleus was demonstrated. In a later paper La Forge (1928) showed that the pyridyl ring is of greater toxicological import than the pyrrolidine system. He prepared several analogues in which this latter ring was opened and found that several of his compounds were equitoxic to nicotine.

More recent work has verified the important pharmacological role of the pyridine nucleus. Haglid and Wellings and their co-workers (1963a, b, c, d) systematically examined the physiological activities of all the pyridylalkylamines associated with carbon-carbon cleavages of the pyrrolidine ring of nicotine, as well as several related analogues. From the great volumes of information presented it would appear that the side chain nitrogen

should, for stimulant activity be methyl substituted, and further, that the other alkyl group should not be larger than an ethyl group. These conclusions are very similar to the earlier mentioned effects of replacing the N-methyl groups of acetylcholine by larger alkyl units. It is unfortunate that Haglid and Wellings did not include estimations of dissociation constants in their communications, for such inclusions would improve the quantitative data by permitting the establishment of ionic concentrations related to the nicotinium ion. However, their researches proved that some pyridylalkylamines are capable of expressing quite a measurable nicotine-like activity. As even these activities were much lower than their standard, nicotine, they concluded that, "the intact pyrrolidine ring is a definite contributing factor to the biological activity of nicotine". Although this statement cannot be specifically denied it must not be assumed that equivalent nicotinic activities cannot be demonstrated by simpler, related substances. The effects of some isomers and analogues of nicotine on junctional transmission were studied by Barlow and Hamilton (1962a). On a series of pharmacological preparations they found some tertiary and quaternary pyridylalkylammonium salts to be several times more active than nicotine, on a molecular or ionic basis. In addition, they were the first to demonstrate

that appreciable nicotinic activity may be associated with gamma-substituted pyridylalkylamines.

Structure-activity relationships--on  
relating nicotine to acetylcholine

The effects on activity evinced by changing the alkylamino substituents of nicotine and of acetylcholine, and the demonstrated activity-dependence of the former on its 'quaternary' ion denotes a prime similarity between the two molecules. The question then arises, what other features may they have in common, of importance to a drug-receptor interaction? Certainly the pyrrolidino nucleus may be suitably modified without necessarily depressing activity, so the question is best answered by examining the pyridine nucleus and comparing this to the acetoxymethylene system of the chemical transmitter. No unequivocal, excessively obvious points of similarity may be arrayed, but there are some tempting theories that can be considered in the attempt to demonstrate equivalence.

The general shape of a pyridine ring is very similar to a benzene ring, and perhaps this is of importance. There is evidence to show that benzyltrimethylammonium is capable of nicotinic activity and as mentioned earlier (Raventos, 1937) this may be correlated to simple salt (n-butyltrimethylammonium or n-propyltrimethylammonium) analogues of acetylcholine.



If beta-pyridylmethyiltrimethylammonium can withstand a similar analysis, an indirect though consecutive thread could satisfactorily unite nicotine and acetylcholine. The beta positions of a pyridine ring are the most 'aromatic' in character (that is the attached protons are more equivalent to protons on a benzene ring), for electrophilic reactions on pyridine affords mainly 3- or 3,5- substituted products (see for example, Katritzky and Lagowski, 1960) and hence there are no incisive chemical objections to this correlation. Pharmacologically there are serious restrictions, for on the frog rectus abdominis preparation benzyltrimethylammonium was found to be somewhat less active than this pyridylalkyl salt (Hamilton and Rubinstein, 1968), and there is further evidence to indicate dissimilarity. Haglid and Wellings (1963a) synthesised the phenyl derivative of nicotine and it proved to be one two-hundredth as active as the tobacco alkaloid. Therefore, a beta-substituted pyridyl is not reconcilable with a phenyl moiety as far as nicotinic activity is concerned.

From molecular orbital theory Longuet-Higgins and Coulson (1947) were able to assign relative electron densities to the atoms of the pyridine ring. On this relative basis the nitrogen is negatively charged and the carbons are positively charged (with the

alpha- and gamma- positions being some three times more positive than the beta-). These assignments, though in no way absolute, are experimentally supportable, for the alpha- positions (and to a lesser extent the gamma-position) can be readily attacked by nucleophilic reagents (for example, the formation of 2- and 4-aminonicotines from nicotine and sodamide (Tschitschibabin and Kirssanow, 1924), and dipole moment estimations of pyridine indicate a positively charged gamma position (Katritzky and Lagowski, 1960). It is possible to regard the pyridine ring as the source of a positive charge in an equivalent position to the ether oxygen in acetylcholine and justify Hey's presumption (Hey, 1949; Barlow, 1965) or satisfy the 'essential negative charge' proponents (Sekul and Holland, 1961a) by invoking the pyridine nitrogen atom. Considerations of charge distribution therefore aids the correlation of nicotine to acetylcholine whatever charge theory is preferred and adopted.

#### Structure-activity relationships-- the specificity of nicotine

As with muscarine, nicotine shows the ability to mimic some of acetylcholine's physiological roles and not affect others, and hence these specificities must reflect some basic differences between the acetylcholine receptors

at the 'nicotinic' and 'muscarinic' sites. It would be exceedingly useful to understand these differences in detail for autonomic drugs could then be tailored to a required purpose. However, our present stage of knowledge in these matters is small and we have but a very limited picture of the receptor system itself. Nonetheless, certain structural features do appear to lead to 'muscarinic' reactions and as these have been briefly examined it remains to treat 'nicotinic' specificity in a like manner.

The relatively widely varying structural features that can present nicotinic activity and the basic difficulty in relating nicotine to acetylcholine are major reasons why this task is not a simple one. The problem is compounded by the general lack of positive information on autonomic drug specificity reported in the literature. Moreover, nicotinic activity can be regarded in two ways. It may possibly arise by default in a biologically active molecule that cannot accommodate more rigid muscarinic requirements, or else a molecule may be truly nicotinic by virtue of its ability to discriminate between basic receptor differences. This somewhat philosophical point makes it difficult to establish true specificity, but the differences may be explained by example. Benzyltrimethylammonium is muscarinic and nicotinic (Hunt, 1926). The molecule may be satisfactorily

related to acetylcholine hence these activities are reasonable. The existence of the "methylene neck" (Cavalitto and Grey, 1960) presupposes muscarinic activity. Extending the molecule to its homologue, phenylethyltrimethylammonium, produces nicotinic activity, and this may reflect a general indisposition to meet muscarinic requirements, whereas complexing occurs with the less critical nicotinic receptor. Phenylethyltrimethylammonium could therefore belong to the former division of 'nicotinomimetics', perhaps nicotine represents the latter. The pyridylmethyltrimethylammonium compounds studied by Hamilton and Rubinstein (1968) were also muscarinic and nicotinic in activities, but a cyclic pyrrolidine analogue, nicotine itself, is pharmacologically virtually nicotine-like.

Assuming that nicotine's specificity is dependent on its receptor differentiating ability, the disturbance of this property with abolition of the pyrrolidine ring is worthy of note. The ring confers optical-activity to the nicotine molecule but this does not, as is the equivalent case with muscarine and its stereo-isomers, greatly modify biological activity. Domino (1965) has reviewed the literature pertaining to stereo-isomers of nicotine and discussed the earlier toxicological researches of Pictet and Rotschy (1904), Macht and

Davis (1934), and Hicks and his associates (Hicks, Brücke and Heuber, 1935; Hicks, Mackay and Sinclair, 1947). These communications reveal the (-)-nicotine is equally, or more active, than its enantiomer, but the optical purity in these works is in doubt. Barlow and Hamilton (1965) and Barlow (1965) have more recently examined the stereo-specificity for nicotinic activity and although the naturally occurring (-)-nicotine is somewhat more active than (+)-nicotine the difference is of low magnitude and is perhaps related to some slightly adverse molecular bulk interference. This view is supported by the general lack of stereo-specificity demonstrated by acetyl- $\alpha$ -methylcholine (Lesser, 1965). Hence nicotinic activity might be as influenced by a suitable alpha substituent as muscarinic activity is by a beta group, but no definitive experiments have, as yet, been performed (cf., Smissman, Nelson, La Pidus and Day, 1966). Such a theory could also accommodate the results of Archer, Lands and Lewis (1962) who showed 2-acetoxytropanes to be potently nicotinic yet only weakly muscarinic-like.

## III. MATERIALS

- A. The following drugs were employed in the determinations of synaptic activity:
- (a) carbamylcholine chloride (Carbachol, B.P.),  
Molecular Weight 182.6  
Supplied by British Drug Houses (Canada), Toronto,  
lot number 115892.
  - (b) benzyltrimethylammonium bromide,  
Molecular Weight 230.2  
Synthesized and kindly donated by Dr. R. B. Barlow,  
Department of Pharmacology, University of  
Edinburgh.
  - (c) phenylethyltrimethylammonium bromide,  
Molecular Weight 244.3  
Synthesized and kindly donated by Dr. R. B. Barlow,  
Department of Pharmacology, University of  
Edinburgh.
  - (d) phenylpropyltrimethylammonium bromide,  
Molecular Weight 258.4  
Synthesized and kindly donated by Dr. R. B. Barlow,  
Department of Pharmacology, University of  
Edinburgh.
  - (e) alpha-pyridylmethyltrimethylammonium bromide  
hydrobromide,  
Molecular Weight 312.1  
Melting Point 192°-194°C.  
Synthesized by the author.  
Analysed as a dipicrate salt. Melting point 174°C.  
Calculated: C. 41.4%, H. 3.31%, N. 18.4%  
Found: C. 41.1%, H. 3.16%, N. 18.3%

- (f) **alpha-pyridylethyltrimethylammonium bromide hydrobromide,**  
Molecular Weight 326.1  
Melting Point 195°-196°C.  
Synthesized by the author.  
Analysed as a dipicrate salt. Melting point 156°C.  
Calculated: C. 42.1%, H. 3.56%, N. 18.0%  
Found: C. 42.6%, H. 3.98%, N. 18.0%
- (g) **alpha-pyridylpropyltrimethylammonium bromide hydrobromide,**  
Molecular Weight 340.1  
Melting Point 176°-178°C.  
Synthesized by the author.  
Analysed as a dipicrate salt. Melting point 190°C.  
Calculated: C. 43.4%, H. 3.80%, N. 17.6%  
Found: C. 43.5%, H. 4.06%, N. 17.5%
- (h) **beta-pyridylmethyltrimethylammonium bromide hydrobromide,**  
Molecular Weight 312.1  
Melting Point 199°-201°C.  
Synthesized by the author.  
Analysed as a dipicrate salt. Melting point 212°C.  
Calculated: C. 41.4%, H. 3.31%, N. 18.4%  
Found: C. 41.6%, H. 3.21%, N. 18.5%
- (i) **beta-pyridylethyltrimethylammonium bromide hydrobromide,**  
Molecular Weight 326.1  
Melting Point 242°-244°C.  
Synthesized by the author.  
Analysed as a dipicrate salt. Melting point 187°C.  
Calculated: C. 42.1%, H. 3.56%, N. 18.0%  
Found: C. 42.2%, H. 3.47%, N. 17.8%
- (j) **beta-pyridylpropyltrimethylammonium bromide hydrobromide,**  
Molecular Weight 340.1  
Melting Point 210°-213°C.  
Synthesized by the author.  
Analysed as a dipicrate salt. Melting point 146°C.  
Calculated: C. 43.4%, H. 3.80%, N. 17.6%  
Found: C. 43.1%, H. 4.03%, N. 17.8%

- (k) gamma-pyridylmethyltrimethylammonium bromide hydrobromide,  
Molecular Weight 312.1  
Melting Point 223°-224°C.  
Synthesized by the author.  
Analysed as a dipicrate salt. Melting point 237°C.  
Calculated: C. 41.4%, H. 3.31%, N. 18.4%  
Found: C. 41.2%, H. 3.26%, N. 18.5%
- (l) gamma-pyridylethyltrimethylammonium bromide hydrobromide,  
Molecular Weight 326.1  
Melting Point 247°-250°C.  
Synthesized by the author.  
Analysed as a dipicrate salt. Melting point 153°C.  
Calculated: C. 42.1%, H. 3.56%, N. 18.0%  
Found: C. 42.2%, H. 3.58%, N. 18.5%
- (m) gamma-pyridylpropyltrimethylammonium bromide hydrobromide,  
Molecular Weight 340.1  
Melting Point 220°-221°C.  
Synthesized by the author.  
Analysed as a dipicrate salt. Melting point 199°C.  
Calculated: C. 43.4%, H. 3.80%, N. 17.6%  
Found: C. 43.7%, H. 3.99%, N. 17.7%
- (n) nicotine hydrogen tartrate,  
Molecular Weight 493.4  
Supplied by British Drug Houses, Poole, England,  
lot number 450660.
- (o) atropine sulphate,  
Molecular Weight 964.8  
Supplied by British Drug Houses (Canada), Toronto,  
lot number 17094.
- (p) hexamethonium dibromide,  
Molecular Weight 362.2  
Supplied by K and K Laboratories, Inc.,  
Plainsview, New York,  
lot number 42754L.
- (q) 2-furfuryltrimethylammonium iodide (Furmethide,  
S.K.F. No. 99)  
Molecular Weight 267.1  
Kindly donated by Smith, Kline and French,  
Montreal, Quebec.



- (r) 3-furfuryltrimethylammonium bromide,  
Molecular Weight 220.1  
Melting Point  $> 300^{\circ}\text{C}$ .  
Synthesized by the author.  
Analysed as a picrate salt. Melting point  $228^{\circ}\text{--}229^{\circ}\text{C}$ .  
Calculated: C. 45.6%, H. 4.38%, N. 15.2%  
Found: C. 45.9%, H. 4.45%, N. 15.0%
- (s) 1-phenylethyltrimethylammonium bromide,  
Molecular Weight 244.2  
Melting Point  $190^{\circ}\text{--}191^{\circ}\text{C}$ .  
Synthesized by the author.  
Analysed as a picrate salt. Melting point  $150^{\circ}\text{C}$ .  
Calculated: C. 51.9%, H. 5.38%, N. 14.2%  
Found: C. 51.6%, H. 5.23%, N. 14.5%
- (t) 2-tetrahydrofurfuryltrimethylammonium bromide,  
Molecular Weight 224.1  
Melting Point  $213^{\circ}\text{--}215^{\circ}\text{C}$ .  
Synthesized by the author.  
Analysed as a picrate salt. Melting point  $123^{\circ}\text{C}$ .  
Calculated: C. 45.2%, H. 5.41%, N. 15.0%  
Found: C. 45.4%, H. 4.97%, N. 15.0%
- (u) pilocarpine hydrochloride,  
Molecular Weight 244.7  
Supplied by British Drug Houses (Canada), Toronto,  
lot number 907711.

Analyses were performed by the Microanalyses  
Laboratory, Toronto, Canada.

B. The chemicals that were used in the syntheses of the  
aforementioned drugs are as follows:

- (a) 2-pyridylmethanol.  
Supplied by Aldrich Chemical Co. Inc.,  
Milwaukee, Wisconsin.
- (b) 2-pyridylethanol.  
Supplied by K and K Laboratories Inc.,  
Plainsview, New York.

- (c) 3-(2-pyridyl)-1-propanol.  
Supplied by Aldrich Chemical Co., Inc.,  
Milwaukee, Wisconsin.
- (d) 3-pyridylmethanol.  
Supplied by Aldrich Chemical Co., Inc.,  
Milwaukee, Wisconsin.
- (e) 3-pyridylacetic acid.  
Supplied by Aldrich Chemical Co., Inc.,  
Milwaukee, Wisconsin.
- (f) 3-(3-pyridyl)-1-propanol.  
Supplied by Aldrich Chemical Co., Inc.,  
Milwaukee, Wisconsin.
- (g) 4-pyridylmethanol.  
Supplied by K and K Laboratories, Inc.,  
Plainsview, New York.
- (h) 4-pyridylethanol.  
Supplied by K and K Laboratories, Inc.,  
Plainsview, New York.
- (i) 3-(4-pyridyl)-1-propanol.  
Supplied by Aldrich Chemical Co., Inc.,  
Rochester, New York.
- (j) trimethylamine hydrochloride.  
Supplied by Eastman Organic Chemicals,  
Rochester, New York.
- (k) lithium aluminium hydride.  
Supplied by British Drug Houses,  
Poole, England.
- (l) 2-furfuryl bromide.  
Supplied by K and K Laboratories, Inc.,  
Plainsview, New York.

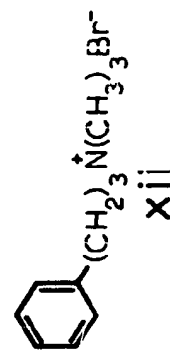
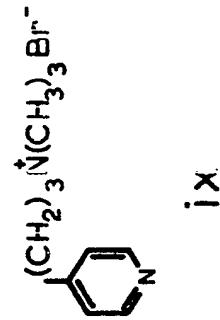
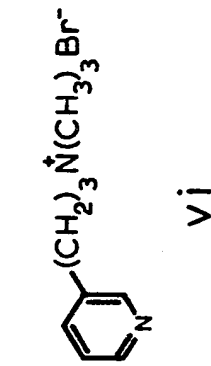
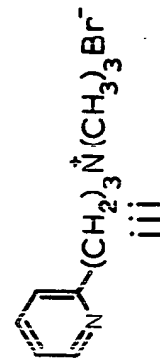
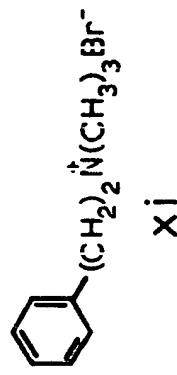
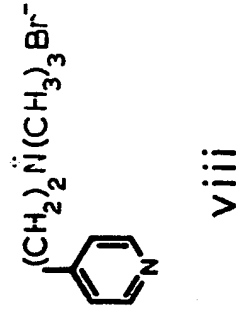
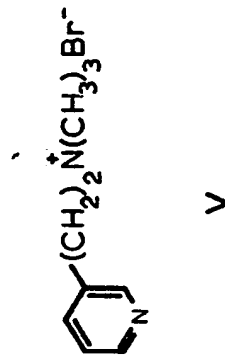
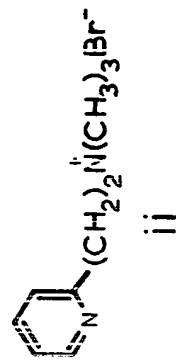
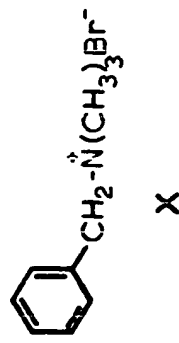
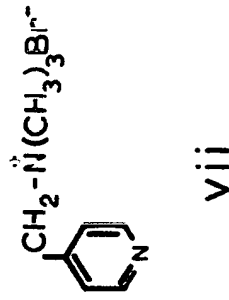
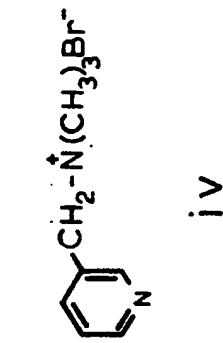
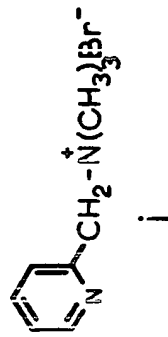
- (m) acetophenone.  
Supplied by Fischer Scientific Company,  
Fair Lawn, New Jersey.
- (n) trimethylamine.  
Supplied by Eastman Organic Chemicals,  
Rochester, New York.
- (o) sodium borohydride.  
Supplied by British Drug Houses,  
Poole, England.
- (p) "Rexyn 201" (Cl-SO<sub>4</sub>, analytical grade).  
Supplied by Fisher Scientific Co.,  
Fair Lawn, New Jersey.
- (q) 3,4-furandicarboxylic acid.  
Supplied by Aldrich Chemical Co., Inc.,  
Milwaukee, Wisconsin.

Figure I

Pyridylalkyl- and Phenylalkyltrimethylammonium Salts

|      |   |                                      |
|------|---|--------------------------------------|
| i    | - | alpha-pyridylmethyltrimethylammonium |
| ii   | - | alpha-pyridylethyltrimethylammonium  |
| iii  | - | alpha-pyridylpropyltrimethylammonium |
| iv   | - | beta-pyridylmethyltrimethylammonium  |
| v    | - | beta-pyridylethyltrimethylammonium   |
| vi   | - | beta-pyridylpropyltrimethylammonium  |
| vii  | - | gamma-pyridylethyltrimethylammonium  |
| viii | - | gamma-pyridylethyltrimethylammonium  |
| ix   | - | gamma-pyridylpropyltrimethylammonium |
| x    | - | benzyltrimethylammonium              |
| xi   | - | phenylethyltrimethylammonium         |
| xii  | - | phenylpropyltrimethylammonium        |

FIG. 1



$\alpha$ -Pyridyl Series

$\beta$ -Pyridyl Series

$\gamma$ -Pyridyl Series

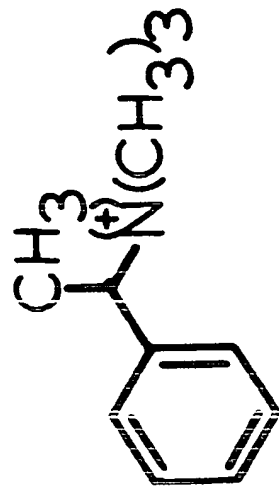
Phenyl Series

Figure 1a

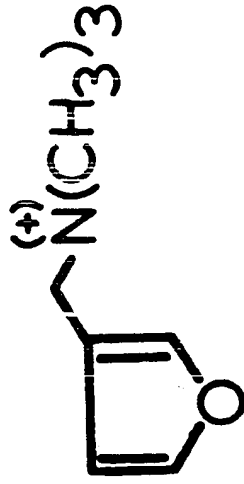
Furfuryl and Benzyltrimethylammonium Analogues

- I - 1-phenylethyltrimethylammonium
- II - 2-tetrahydrofurfuryltrimethylammonium
- III - 3-furfuryltrimethylammonium
- IV - 2-furfuryltrimethylammonium

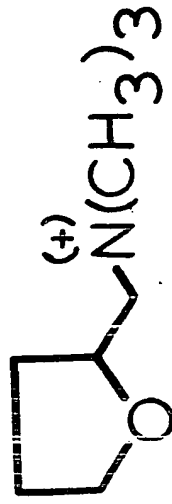
FIG. 1a



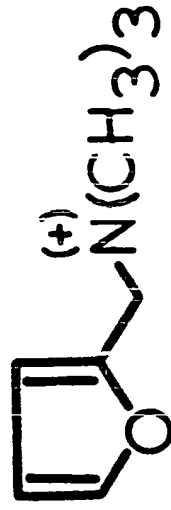
i



iii



ii



iv

## IV. METHODS

The object of this study was to investigate some peripheral cholinergic activities of the following compounds; benzyltrimethylammonium (phenylmethyltrimethylammonium), phenylethyltrimethylammonium, phenylpropyltrimethylammonium and the analogous alpha-, beta- and gamma-pyridylalkyltrimethylammonium salts (figure 1). In addition the equivalent synaptic properties of 1-phenylethyltrimethylammonium, 2-furfuryltrimethylammonium, 3-furfuryltrimethylammonium and 2-tetrahydro-furfuryltrimethylammonium (figure 1a) were examined. The bioassay methods used included the isolated frog rectus abdominis muscle preparation, the isolated phrenic nerve-diaphragm preparation, and the isolated guinea-pig ileum preparation. The compounds illustrated in figure 1 were also assayed on the unanaesthetised chick. The isolated frog rectus abdominis and guinea-pig ileum experiments, used for quantitative potency determinations on the studied compounds, were automated to minimise any experimental bias. In all the isolated muscle experiments carbamylcholine (Carbachol) was used as the standard



reference substances against which the other compounds were assayed. However, an assay of pilocarpine on the hexamethonium treated guinea-pig ileum was performed to provide an additional point of comparison for the "muscarinic" determinations.

The synthesis of the pyridylalkyltrimethylammonium salts is described as a general method for the formation of these compounds from the equivalent pyridylmethyl, pyridylethyl and pyridylpropyl alcohols. The remaining drugs, bromide salts of 3-furfuryltrimethylammonium, 1-phenylethyltrimethylammonium and 2-tetrahydrofurfuryltrimethylammonium were prepared by closely related synthetic methods.

### Bioassay Experiments

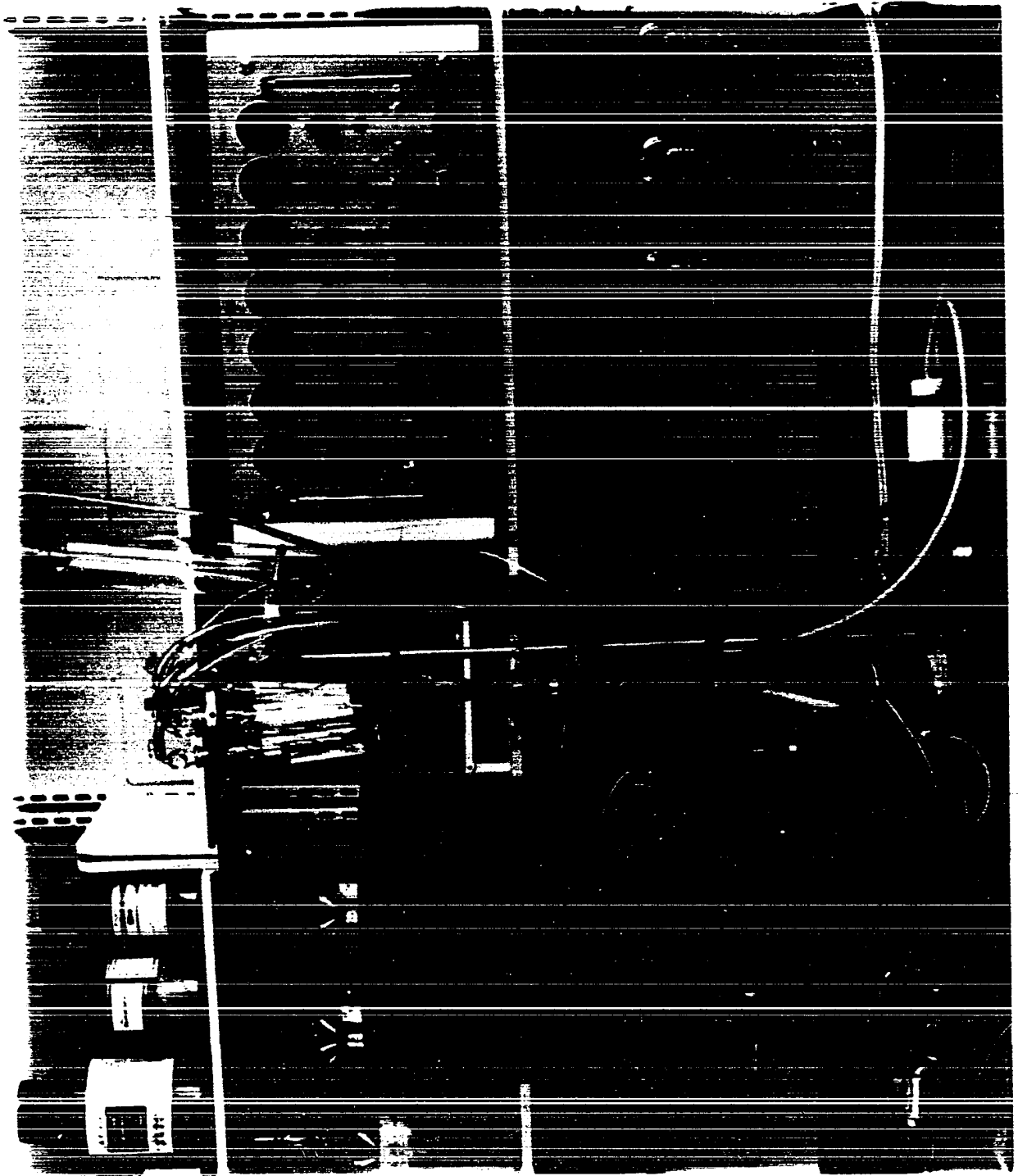
#### Experiments on the rectus abdominis muscle of the frog (*Rana pipiens*)

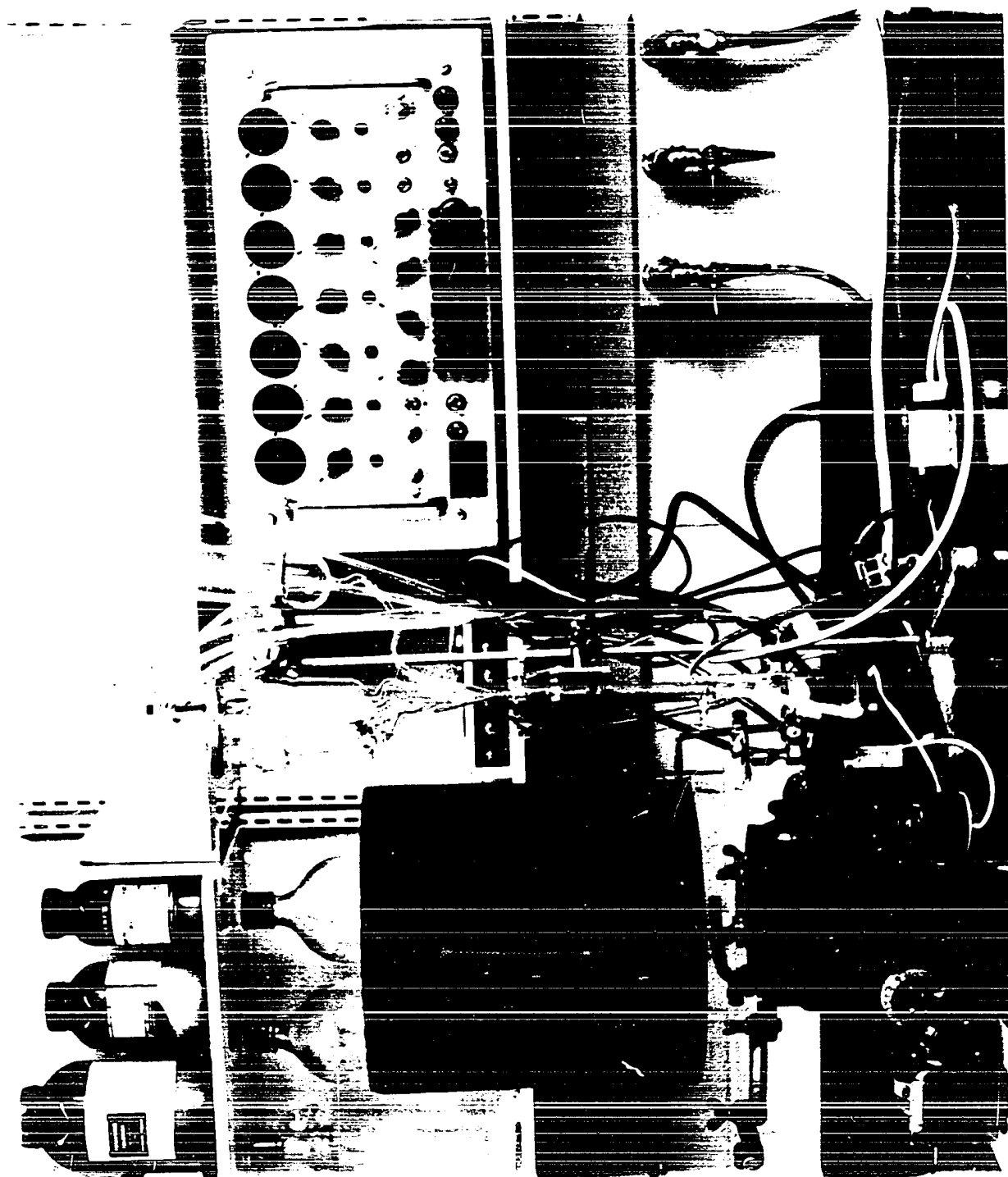
##### A. Equipotent molar ratios

A frog was stunned and decapitated and the spinal cord destroyed by pithing. The rectus abdominis muscle was dissected out and the muscle transferred to a dish containing Clark's modified Ringer solution at room temperature. The muscle was then mounted in a 5ml aerated organ bath containing this solution and the thread tied to a gimbal lever (Palmer) and balanced. The muscle was stretched for a period of thirty minutes prior to use. The

Figure 2

Apparatus used in the automatic bioassay determinations on the frog rectus abdominis and the guinea-pig ileum. The flow of solutions into the organ bath is controlled by air-valves which are opened at selected intervals by the timing and selector unit. This unit additionally permits the order of applied drug solutions to be chosen for the particular experiment.





automated apparatus (figure 2) consisted of a Vickers' "Automatic Bioassay Apparatus" which timed and performed the following operations:

1. Time, zero. Muscle stretched (2g weight) in aerated Clark's Ringer Solution.
2. Time, 2 minutes. Weight elevated by air-pump pressure thereby allowing muscle to contract slightly and stabilise. The drum was also turned on and a base line established. Mechanical vibration was provided by an electric motor and cam during the period in which the drum was on.
3. Time, 3 minutes. Bath emptied and refilled by fresh Ringer solution. Then bath emptied again and a solution of drug in Clark's Ringer solution was added from the elevated reservoirs. The resulting contracture was recorded on the smoked paper.
4. Time, 4.5 minutes. Drum and air pump turned off thereby delineating the contracture and reapplying the stretching 2g weight. This was followed by the drug solution being emptied and the muscle being washed twice by fresh Ringer solution, subsequent to which a further

portion of this solution was added for the stretching-recovery period of 1 1/2 minutes.

All doses of drugs were established prior to automation. Solutions of the drugs were added directly to the muscle and contractures recorded for 90 seconds. In each experiment carbamylcholine was used as the reference compounds and one other substance was assayed for activity against it. Four-point assays were performed with two dose levels of the standard compound  $S_1$  and  $S_2$  and two dose levels of the unknown compound,  $U_1$  and  $U_2$  (where  $S_1/S_2 = U_1/U_2$ ) being administered such that the resulting contractures were similar in size between the dose levels of the two drugs. These contractures were also of a size considered to lie between 20% and 80% of a maximum contracture (frequently but not in all cases confirmed at the end of the experiment by applying a concentrated ( $1 \times 10^{-3}M$ ) solution of carbamylcholine. As there was great experimental consistency, this was not performed in all cases). The order of addition of the drug solutions was based on a "Latin Square" design using not less than a total of 16 doses. On completing the bioassay, the smoked paper was fixed with shellac solution. The contractures were measured (as m.m. response) on the response limiting curve (formed when the drum switched off and the weight re-applied), and the equipotent molar ratio (E.P.M.R.) determined by graphical means, or by the

method used by Gaddum (1953, 1959) for a similar experimental design. Each compound was thus bioassayed several times and a final E.P.M.R.  $\pm$  S.E.M. (standard error of the mean) noted.

B. Maximum contractures produced by the drugs

The method employed to investigate whether or not the compounds could produce a maximum contracture of the muscle was somewhat related to that described by Ariens (1954).

The muscle was mounted in a 5ml organ bath as above and doses of drugs added that produced maximal responses, recorded as contractures scribed on the drum. Following attainment of the maximal contracture, the drum was turned on for two minutes and the height above a pre-determined base line obtained. Following this period, the drum was stopped, a stretching weight applied to the muscle and the drug washed out. After a resting period of half an hour the weight was removed and a new steady base line established. The process was then repeated. Each drug was tested against carbamylcholine and each dose was repeated in alternating order a minimum of three times. Carbamylcholine was also tested against itself to achieve a true standard against which to compare the agonistic behaviour of the other substances. The heights of the

drug-induced contractures (m.m. response) were determined, and averaged, and compared with the like responses from carbamylcholine. Pilot tests were performed to determine the concentrations of drug required to obtain maximum contractures before the assays.

Experiments with the phrenic  
nerve-diaphragm of the rat

A male or female albino rat was sacrificed by a blow on the head and quickly exsanguinated. The diaphragm was exposed and a fan-shaped segment dissected out (Bülbring, 1946). This was gently lifted and the phrenic nerve was then carefully freed from most of the attached membrane as far as the thymus. The nerve was then severed below the thymus and the preparation transferred to a dish containing Krebs' bicarbonate buffer solution.

A thread was then securely tied to the tendinous apex and the membrane attached to the nerve cut near the muscle (so subsequent contractions of the muscle would not displace the nerve from the fluid electrode). The ribs were then impaled on the mounting pins and partially lowered into the water-jacketed 50ml organ bath. (The temperature of the bath and the immediate reservoir was maintained at 37°C by circulating warm water through the jackets that enclosed these pieces of apparatus.) The thread was then tied to the semi-isometric torsion lever



(Condon, 1957). The preparation was then fully lowered into the well gassed (95% oxygen 5% carbon dioxide) bathing medium (Krebs' bicarbonate buffer solution) and the thread tension adjusted. The freely floating nerve was then drawn into the fluid electrode (Furshpan and Potter, 1959).

The nerve was stimulated 12 times per minute by a rectangular-wave pulse of 0.7 milli-seconds duration, the voltage applied being twice that required to produce a maximum contraction. (The stimulator was a Grass Instrument Company S5 model.) After the preparation had steadied and the twitches on the smoked drum were uniform, the bioassay was begun.

Drugs were pipetted into the bath from stock solutions made up in saline according to a "Latin Square" design, with a minimum of 16 doses being used. The time cycle adopted was as follows:

1. Time, zero. Stimulant impulses were continued in the absence of drug. The drum was off.
2. Time, 5 minutes. The drum was turned on and a recording made in the absence of drug.
3. Time, 6 minutes. The drug was administered and the drum switched off. Impulses were continued.

4. Time, 11 minutes. The drum was turned on and the depressed twitches recorded.
5. Time, 12 minutes. The drum switched off, the drug drained out and the preparation washed with fresh warm Krebs' buffer solution.

The cycle was then repeated.

On completing the bioassay, the smoked paper was fixed with shellac solution and after drying, measurements of blocking action were made. This involved estimating the percentage reduction in height between the contractions before drug administration and after the attainment of the drug induced 'twitch' plateau. These figures were used in the subsequent determination of equipotent molar ratio by the previously described methods.

#### Experiments with the guinea-pig ileum

A guinea-pig was killed by a blow on the head. Its throat was cut and it was quickly exsanguinated. The abdomen was opened and a length of ileum was freed of mesenteric attachments and, after cutting out, was transferred to a dish containing Tyrode's solution. The contents of the ileum were washed out by placing the duodenal end over the tip of a pipette containing Tyrode's solution and tilting the pipette slightly to apply a small

hydrostatic pressure. A portion of ileum 2-3 cm in length was then cut from the caecal end and transferred to a second dish containing Tyrode's solution. (If the ileum was not excessively full, another method of cleaning was used. The required piece of ileum was removed to the second dish and a solution of carbamylcholine ( $1 \times 10^{-5} \text{M}$ ) in Tyrode's solution dropped onto the specimen. The resulting contracture generally cleared the lumen.) Needles were then passed through the wall at either end of the piece of ileum and the bi-threaded ileum mounted in the aerated, 2ml water jacketed, organ bath. (Water was circulated through this jacket and through a jacketed drug and wash addition port, thus maintaining the smooth muscle preparation at  $37^{\circ}\text{C}$ .) A thread was tied to a frontal writing level and after applying a stretching tension, the lever was carefully balanced. As the preparation was required to produce "muscarinic" responses only, the plexal (parasympathetic) ganglia had to be blocked. This was achieved by replacing the Tyrode's solution used for washes by 'Tyrode's/Hexamethonium' solution. This was prepared by diluting 10ml of  $10^{-2} \text{M}$  hexamethonium bromide solution in saline to one litre with Tyrode's solution. (It was found experimentally that this  $1 \times 10^{-4} \text{M}$  hexamethonium solution could block the stimulant action of  $2 \times 10^{-5} \text{M}$  nicotine.) Pilot tests were then conducted to establish the concentrations of

drugs to apply for the bioassay. Drug solutions were prepared in the modified Tyrode's solution. It was found to be advantageous to keep the preparation functioning after establishing a cycle of drug addition, wash and rest, the muscle responses becoming much less erratic, affording rapid rising, self-terminating contractions. The drug reservoirs were then filled with the chosen concentrations of carbamylcholine and test compound for the two and two dose assay. Two concentrations of each being used that produced similar sized contractions that lay between 20% and 80% of the maximum (as with the previously described frog rectus abdominis experiments). The apparatus was then placed on the automatic setting and the bioassay proceeded using the following time sequence.

1. Time, zero. The preparation was at rest, the drum was off.
2. Time, 1 minute 15 seconds. The drum was turned on, the bath was drained and a wash added.
3. Time, 1 minute 50 seconds. The bath was drained and the drug added.
4. Time, 2 minutes 40 seconds. The drum was turned off. The bath was drained, the

preparation washed twice and the bath then refilled with fresh, warm bathing solution. These steps took the last 20 seconds in this 3 minute cycle.

The order of drug additions was based on a 4 x 4 "Latin Square". Each compound was assayed several times and equipotent molar ratios calculated. After the bioassays the preparation was frequently treated with nicotine ( $2 \times 10^{-5}M$ ) in Tyrode's/Hexamethonium solution to check that the ganglia were blocked. Similarly, the total abolition of response produced when the drug was administered in the presence of atropine ( $1 \times 10^{-6}M$ ) proved the drugs to be acting at the postganglionic postsynaptic site, and the responses were not mediated, for example, by released serotonin. Further assays were not performed on atropine treated segments of ileum. On occasion the administered test substance would not produce a response in concentrations up to and including  $5 \times 10^{-3}M$ . After several trials to produce a response the drug would be classified as inactive, with an E.P.M.R. of greater than  $2 \times 10^4$ .

Formulae for the above-mentioned physiological solutions are described in Appendix I.

#### Experiments on the unanaesthetised chick

These qualitative experiments were performed

to demonstrate the effects (if any) of the test substances on intact chicks. No attempt was made to assess threshold doses or to judge potency. Healthy, four day old chicks were injected intraperitoneally with 1 ml. of  $1 \times 10^{-2}$  M solutions of the drugs in saline (0.9% w/v), one compound only being tested on each chick. A control chick received an injection of saline alone. The chicks were placed on a bench top to see if the response characteristic of potent depolarising drugs, defined by Buttle and Zaimis (1949) as "a spastic paralysis in which the legs are rigidly extended and the head thrust back", developed in a reasonable time. Representative photographs were taken of the chicks in this position if the response proved positive, or after ten minutes if no such paralysis was observed.

### Chemical Syntheses

#### Syntheses of the pyridylalkyltrimethylammonium bromide hydrobromide salts

The freshly distilled pyridyl alcohol (0.1 mole) was dissolved in chloroform (100ml) and benzene (100ml) in a 500 ml round-bottomed flask surrounded by an ice-bath. A solution of purified thionyl chloride (Vogel, 1956) (0.2 mole) in dry ether (100ml) was added dropwise to the well-stirred pyridyl alcohol solution. After this addition the ice-bath was removed and stirring continued

for a further hour. (The pyridylpropyl alcohol series were treated somewhat differently in that they were gently refluxed for this hour.) Methanol was then added dropwise to decompose excess thionyl chloride. Solvent was then removed on a Buchi Rotavapor 'R' model rotary evaporator and the pale amber viscous liquid induced to crystallise, if it did not do so of its own accord. The pyridylalkyl chloride hydrochloride was then recrystallised to a constant melting point from ethanol or ethanol/ether and after filtration was dried 'in vacuo'.

The pyridylalkyl chloride hydrochloride (2g to 5g) was dissolved in a minimal volume of methanol (5ml to 10ml) and a (20% w/v) methanolic solution of trimethylamine (a 3 times excess) added slowly. The flask was securely stoppered and shaken at room temperature for 48 hours. (The members of the pyridylpropyl chloride hydrochloride series were refluxed in a closed system for 24 hours.) After this time the methanol and excess trimethylamine was roto-evaporated away. The semi-crystalline mass was then redissolved in methanol/water (9:1, 30ml) and slowly percolated through an ion-exchange resin column ("Rexyn" 201, Fisher Scientific Company; 50g to 100g previously prepared in the hydroxide form and then washed free of water with methanol), using methanol as eluent until the percolate was no longer alkaline. Methanol was then distilled away from the

solution (water bath temperature of 50°C) until all the trimethylamine was removed. (This end point was detected by odour and then by suspending a piece of moistened wide-range pH paper ("Accutint", Anachemia Chemical Company) over the surface of the solution. It was found experimentally that this procedure was capable of detecting 10mg of trimethylamine in 1000ml of methanol.) Water (200ml) was added to the flask and the methanol then distilled away completely. The solution was then extracted with ether/chloroform (4:1, 3 x 50ml) to remove non-water soluble material and the aqueous medium then acidified to pH2 with concentrated hydrobromic acid ("Analar" quality, B.D.H.). The water was then distilled off, the last traces being removed azeotropically by ethanol/benzene/or isopropanol and the residual gum treated with various solvents including methanol, ethanol, isopropanol, acetone, methylethyl ketone and diethyl ether, to enable crystallisation to begin. The crude salt so obtained (of variable yield but generally greater than 50%) was then recrystallised from methanol or methanol/ether to a constant melting point. As these salts were hygroscopic they were stored in a desiccator over anhydrous calcium sulphate.

This method was found to give on the moderate scale employed, a cleaner crude salt than that obtained by the procedure described by Barlow and Hamilton (1962).



### Synthesis of 3-pyridylethanol

All the pyridyl alcohols, bar 3-pyridylethanol, were available commercially (see section III). In consequence this required alcohol was prepared from the available 3-pyridylacetic acid by the following method.

3-pyridylacetic acid (20g) was dissolved in benzene (100ml) and methanol (150ml) and gassed with hydrogen chloride. On removing the solvents 3-pyridylacetic acid hydrochloride crystallised out. This was mixed with toluene (100ml), ethanol (300ml) and sulphuric acid (5ml) and refluxed for 24 hours (Micovic, 1937). A Dean and Stark head was then attached and the flask contents reduced to about one quarter. The residual solvent was then rotoevaporated away and the oil dissolved in water (200ml). The pH was reduced to 9 with a saturated solution of sodium carbonate and this solution then extracted immediately with diethyl ether (5 x 100ml). After drying the ether extracts over anhydrous sodium sulphate the solutions were filtered and distilled to afford ethyl 3-pyridylacetate as an oil (boiling point  $130^{\circ}$ - $132^{\circ}$ C/10mm; Barlow and Hamilton, 1962; Protiva, Jilek and Pliml, 1951).

To a well stirred suspension of lithium aluminium hydride (5g) in dry ether (300ml) was slowly added a solution of ethyl 3-pyridylacetate (20g) in dry ether (100ml). On completion of the addition the mixture was

refluxed for 30 minutes, allowed to cool and then the complex destroyed by the dropwise addition of a saturated solution of sodium potassium tartrate. The ether layer was decanted off and the pasty residue extracted with boiling benzene (3 x 200ml). After drying the combined organic extracts over anhydrous sodium sulphate, filtration and distillation yielded the required 3-pyridylethanol. This was distilled prior to chlorination (boiling point 150°C/10mm; Barlow and Hamilton, 1962; Dornow and Schacht, 1947).

#### Synthesis of 3-furfuryltrimethylammonium bromide

The projected synthesis of this drug required the corresponding alcohol, 3-furfuryl alcohol to undergo the chlorination and subsequent amination procedures described for the pyridyl series. Unfortunately this alcohol was not available and it had to be prepared from 3,4-furandicarboxylic acid.

This di-acid was firstly decarboxylated to 3-furanmonocarboxylic acid after the method of Reichstein and co-workers (Reichstein, Grüssner, Schindler and Hardmeirer, 1933) and the mono-acid so formed was reduced to the required alcohol with lithium aluminium hydride.

3,4-furandicarboxylic acid (10g) was added to a suspension of copper chromite (10g) (Adkin and Connor, 1951) in quinoline (50ml), and the mixture boiled for 1 hour.

After this time, the reaction mixture was filtered and, after acidification with dilute hydrochloric acid, extracted with ether (3 x 50ml). The ethereal solution was then dried over sodium sulphate prior to filtration and removal of the solvent. The tan coloured crystalline residue obtained was sublimed and the white sublimate recrystallised from water. The furan-3-carboxylic acid melted at 122-123°C (Reichstein, Grüssner, Schindler and Hardmeirer, 1933).

The conversion of this acid to 3-furfuryl alcohol and the later formation of 3-furfuryl chloride was originally described by Sherman and Amstutz (1950).

Furan-3-carboxylic acid (5g) was dissolved in dry ether (100ml) and added dropwise to a suspension of lithium aluminium hydride (5g) in ether (100ml) at room temperature. After all of the acid solution had been added the reaction mixture was refluxed for two hours. Decomposition of excess reducing agent with dilute hydrochloric acid afforded a clear solution which was then extracted with ether (5 x 100ml). After drying the ethereal solution over sodium sulphate, filtration and removal of the solvent yielded the required alcohol (4.3g) as a colourless water-miscible oil (boiling point 78-80°C/16mm; Sherman and Amstutz, 1950). Conversion of the alcohol to 3-chloromethylfuran was accomplished by treatment with thionyl chloride as

previously described for the pyridyl series. This was not distilled prior to conversion to the alkyl trimethylammonium chloride. Anion exchange on "Rexyn 201" subsequently yielded 3-furfuryltrimethylammonium bromide.

#### Synthesis of 1-phenylethanol

As the above alcohol was a proposed intermediate in the synthesis of 1-phenylethyltrimethylammonium bromide, its preparation was undertaken.

Acetophenone (12g) was dissolved in methanol (50ml) and the solution added slowly to sodium borohydride (6g) in methanol (10ml). The reaction mixture was maintained at room temperature for one hour. Acidification and ether extraction in the usual way afforded the intermediary alcohol, 1-phenylethanol (11.4g). This was converted to the trimethylammonium bromide analogue by the previously described general method.

#### Synthesis of 2-tetrahydrofurfuryltrimethylammonium bromide

As 2-tetrahydrofurfuryl bromide was available commercially, the conversion into 2-tetrahydrofurfuryltrimethylammonium bromide was a one stage process.

A solution of trimethylamine in methanol (15ml,

20% w/v solution) was added to 2-furfuryl bromide (5g). After two days at room temperature, removal of the solvent yielded an amber oil which partially crystallised. This was dissolved in water (100ml) and extracted with benzene (3 x 20 ml). The aqueous solution was taken to dryness and the pale brown crystalline residue decolourised with animal charcoal ("Norit A", British Drug Houses).

Aside from the purity of the investigated drugs evinced by constant melting points a further test for homogeneity was performed on thin-layer chromatographic plates of aluminum oxide (Type E., E. Merck, Darmstadt, Germany). Eluents were either methanol/chloroform (5%/95% v.v.) or methanol/ethanol/chloroform (5%/5%/90% v.v.) and the chromatograms were developed with Draggendorf's reagent.

#### Formation of picrate salts

The picrate salts were prepared by dissolving the drug hydrobromides (30mg-70mg) in warm water (2ml) and adding solutions of sodium picrate (7mg-200mg) in water (3ml-5ml). The aqueous phases were decanted from the resulting crystals or solidified oils. After washing the solids with water they were recrystallised from ethanol/water.

The samples for analysis were dried at 100°C. "in vacuo" (approximately 20 m.m.) for a minimum of 24 hours.

Figure 3

Typical Experimental ResponsesFrog Rectus Abdominis -- Bioassay: (Automatic)

Carbamylcholine ( $1 \times 10^{-5}$  M,  $2 \times 10^{-5}$  M)

$\beta$ -pyridylethyltrimethylammonium ( $2.5 \times 10^{-6}$  M,  $5 \times 10^{-6}$  M)

Drugs were in contact with the muscle for  $1\frac{1}{2}$  minutes.

The small intermediate peaks are due to washes and drainages which occur before application of the drug solution.

Rat Phrenic Nerve-Diaphragm -- Bioassay

Carbamylcholine (1.5  $\mu$ M, 3  $\mu$ M)

$\gamma$ -pyridylmethyltrimethylammonium (0.3  $\mu$ M, 0.6  $\mu$ M)

Supermaximal voltage: (8volts). Duration: 0.7 m/sec.

Rate 12 shocks/min.

The "blocking" of the normal response is illustrated in this portion of a bioassay. The stepped appearance results from the recording drum being switched on for the final minute of the cycle.

Guinea-Pig Ileum -- Bioassay: (Automatic)

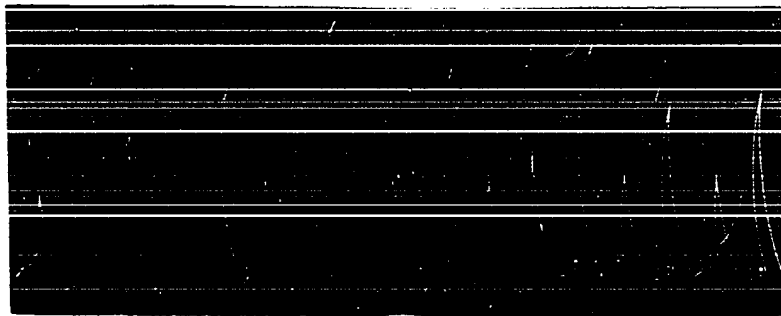
Carbamylcholine ( $1 \times 10^{-7}$  M,  $2 \times 10^{-7}$  M)

$\alpha$ -pyridylmethyltrimethylammonium ( $2 \times 10^{-6}$  M,  $4 \times 10^{-6}$  M)

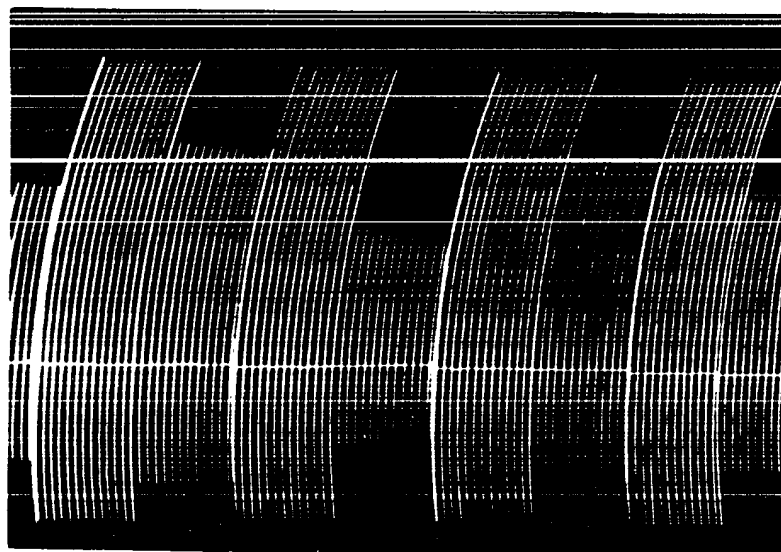
Drugs were in contact with the muscle for 50 seconds.

The small peaks result from the wash and drainage that occur before drug addition.

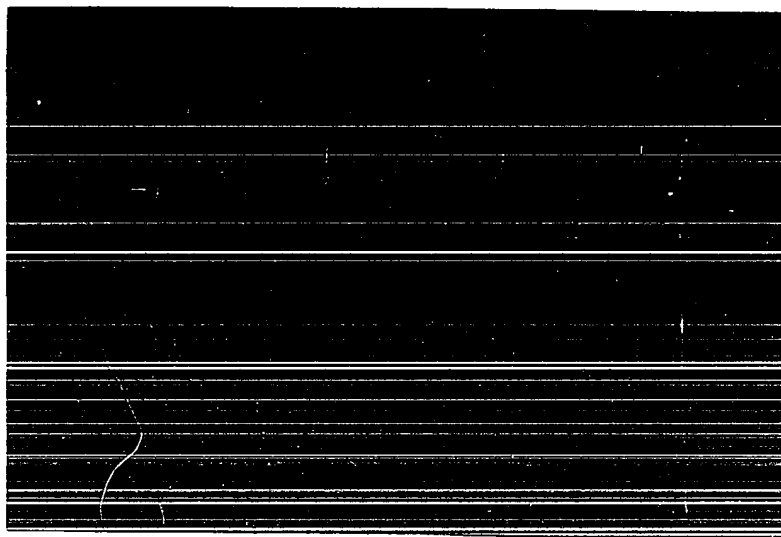
# TYPICAL EXPERIMENTAL RESPONSES



FROG RECTUS ABDOMINIS

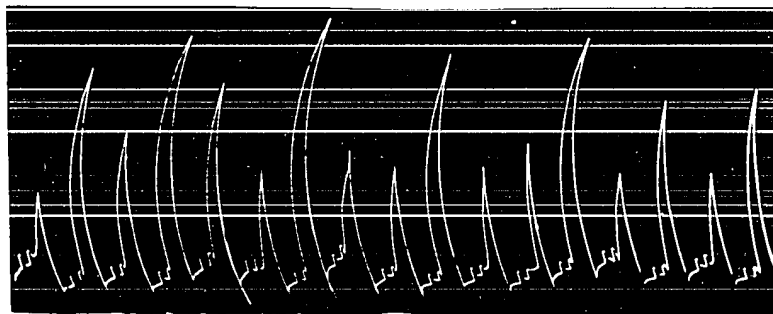


RAT DIAPHRAGM

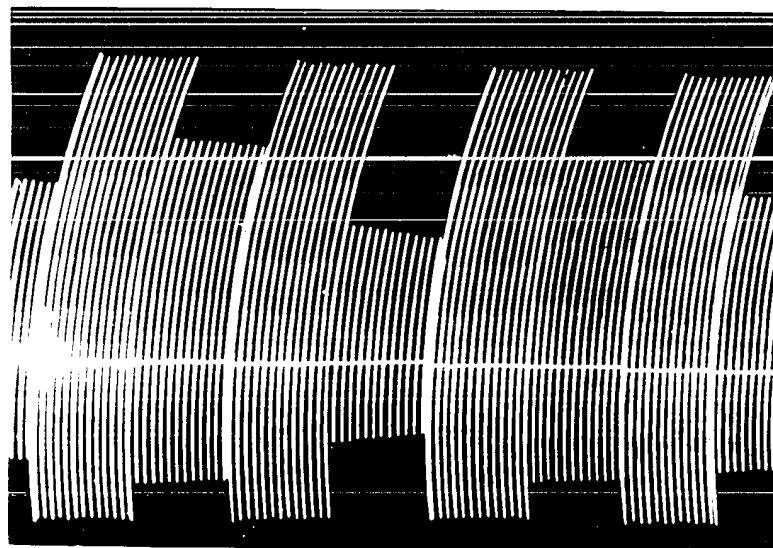


GUINEA-PIG ILEUM

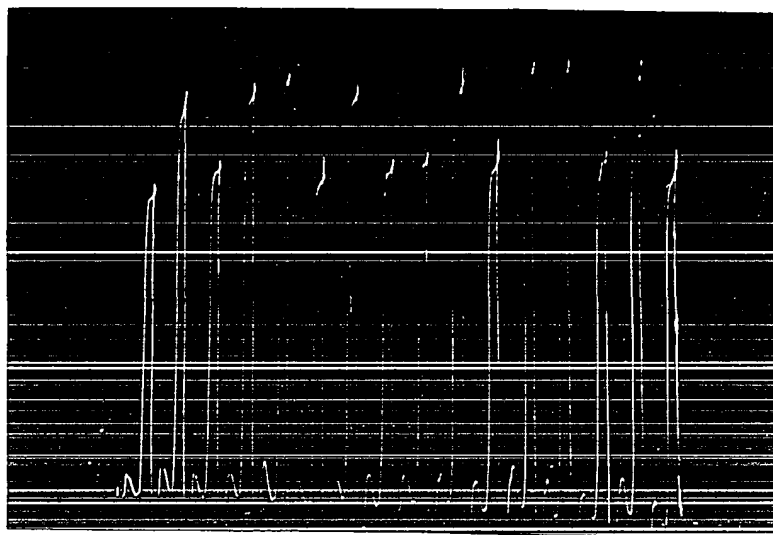
# TYPICAL EXPERIMENTAL RESPONSES



FROG RECTUS ABDOMINIS



RAT DIAPHRAGM



GUINEA PIG ILEUM



## V. RESULTS

The experimental results are presented in the order described in the last section, the relative quantitative activities of drug-synaptic effectiveness being followed by the qualitative study on the whole chick. Isolated muscle responses, obtained during the quantitative determinations, are illustrated in figure 3.

### Quantitative Results

Equipotent molar ratios (E.P.M.R.) have been calculated in order to determine the activity of the test drug when compared to the chosen standard drug. These values indicate the number of quanta of molecules of one substance required to produce the same effect as one quantal molecule of the standard drug. Carbamylcholine chloride (Carbachol) was used as the standard drug in these determinations and therefore was assigned an arbitrary value of unity (1.0). Thus, if the test compound proved to be more active than carbamylcholine the E.P.M.R. would reflect this by being less than 1.0.

Table I  
 Contracture of Frog Rectus Abdominis  
 Pyridylalkyl- and Phenylalkyltrimethylammonium Salts

TABLE 1 - Contracture of Frog Rectus

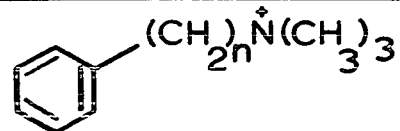
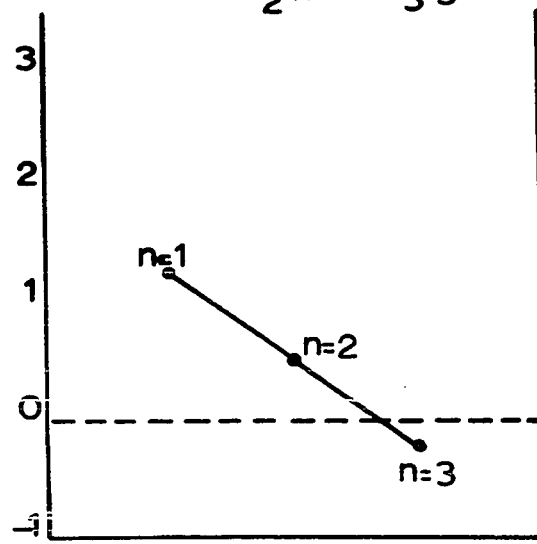
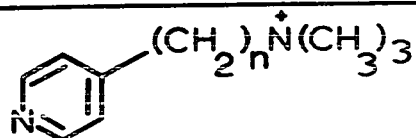
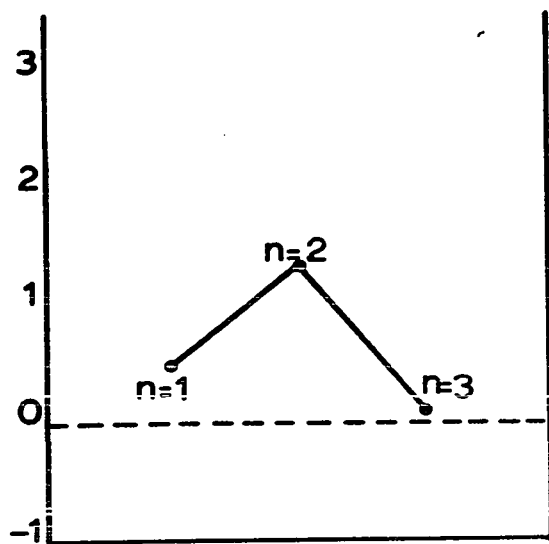
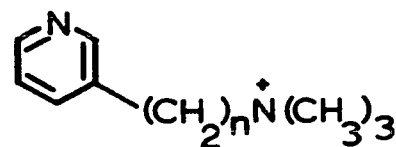
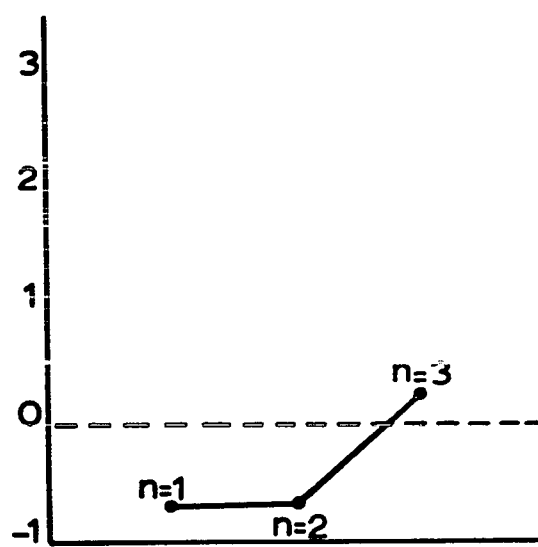
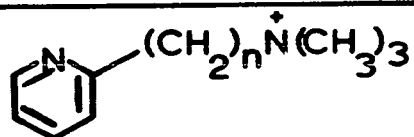
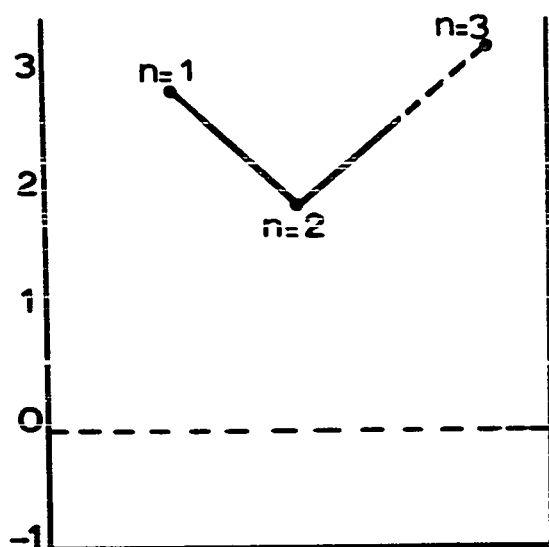
| Compounds                            | E.P.M.R. | ± | S.E.M.* | n= |
|--------------------------------------|----------|---|---------|----|
| beta-pyridylmethyltrimethylammonium  | 0.213    | ± | 0.0071  | 3  |
| beta-pyridylethyltrimethylammonium   | 0.217    | ± | 0.0167  | 4  |
| phenylpropyltrimethylammonium        | 0.749    | ± | 0.025   | 3  |
| carbamylcholine                      | 1.00     |   |         |    |
| gamma-pyridylpropyltrimethylammonium | 1.33     | ± | 0.071   | 3  |
| beta-pyridylpropyltrimethylammonium  | 2.26     | ± | 0.044   | 3  |
| gamma-pyridylmethyltrimethylammonium | 3.28     | ± | 0.087   | 4  |
| phenylethyltrimethylammonium         | 3.46     | ± | 0.113   | 3  |
| benzyltrimethylammonium              | 20.53    | ± | 1.68    | 3  |
| gamma-pyridylethyltrimethylammonium  | 21.4     | ± | 0.68    | 3  |
| alpha-pyridylethyltrimethylammonium  | 94.8     | ± | 2.88    | 3  |
| alpha-pyridylmethyltrimethylammonium | 774.3    | ± | 68.15   | 3  |
| alpha-pyridylpropyltrimethylammonium | >2000    | ± |         | 2  |

\*

Mean results arranged in order of decreasing potency.  
n= number of experiments.

Figure 4  
Frog Rectus Abdominis  
 $\text{Log}_{10}$  Equipotent Molar Ratios  
Pyridylalkyl- and Phenylalkyltrimethylammonium Salts

FIG. 4  
Frog Rectus Abdominis  
Log<sub>10</sub> Equipotent Molar Ratios



and conversely, the E.P.M.R. of a less active drug would be greater than 1.0. This perhaps confusing situation accords with experimental practise, less material being required of more active compounds and vice versa.

In figures 4, 7 and 9, the logarithm (base 10) of the E.P.M.R. is used. Hence carbachol with its value of 1.0 is represented by the log-scale 0.0. When intra-series comparisons between phenyl and pyridyl substituted salts are made (figures 5 and 8) the  $\log_{10}$  E.P.M.R. values for the former compounds are similarly placed at 0.0.

Whenever necessary, the student "t" test was employed to determine the possible statistical significance of two means (Croxtan, 1953; Moroney, 1965) and limits of significance were accepted at or below the 5% level ( $P \leq 0.05$ ).

#### Frog Rectus Abdominis--Equipotent Molar Ratio Determinations

The relative activities of the test compounds are summarised in figure 4 and tables 1 and 1a.

It is evident from the results presented that beta-pyridylmethyltrimethylammonium and beta-pyridylethylammonium are the most active compounds on this preparation and there is no difference between their activities. They are both between four and five

Table 1a

Contracture of Frog Rectus Abdominis

Furfuryl and Benzyltrimethylammonium Analogues

TABLE 1a - Contracture of Frog Rectus

| Compounds                             | E.P.M.R. |      | S.E.M. |   | n= |
|---------------------------------------|----------|------|--------|---|----|
|                                       | +        | -    | +      | - |    |
| carbamylcholine                       |          | 1.00 |        |   |    |
| 3-furfuryltrimethylammonium           | +        | 4.90 | 0.229  |   | 3  |
| 1-phenylethyltrimethylammonium        | +        | 28.7 | 4.07   |   | 4  |
| 2-tetrahydrofurfuryltrimethylammonium | +        | 31.4 | 1.35   |   | 3  |
| 2-furfuryltrimethylammonium           | +        | 47.5 | 6.22   |   | 3  |

\*\*

Mean results arranged in order of decreasing potency.  
n= number of experiments.



times as active as carbamylcholine. The other compound that proved to be more active than the reference compound was phenylpropyltrimethylammonium. Gamma-pyridylpropyltrimethylammonium appeared to be slightly less active than carbamylcholine, but it is questionable as to whether there is any significant difference between the two ( $0.1 > P > 0.05$ ). The standard drug was more than twice as active as the remaining member of the beta-pyridyl series, beta-pyridylpropyltrimethylammonium and more than three times more active than gamma-pyridylmethyltrimethylammonium and phenylethyltrimethylammonium. There was no significant difference between the latter two compounds. Additionally, carbamylcholine proved to be some twenty times more active than gamma-pyridylethyltrimethylammonium. The alpha-pyridyl series as a whole was far less active than the standard, with its most active member, alpha-pyridylethyltrimethylammonium, being nearly one-hundredth as active as the reference drug.

Although all of the additional drugs assayed on the frog rectus abdominis proved to be less active than the carbamylcholine standard (table 1a), there were differences between the demonstrated activities. The most potent of this group was 3-furfuryltrimethylammonium which possessed approximately one quarter of the activity of carbamylcholine, whereas the lowest potency was recorded for the isomeric 2-furfuryltrimethylammonium ion.

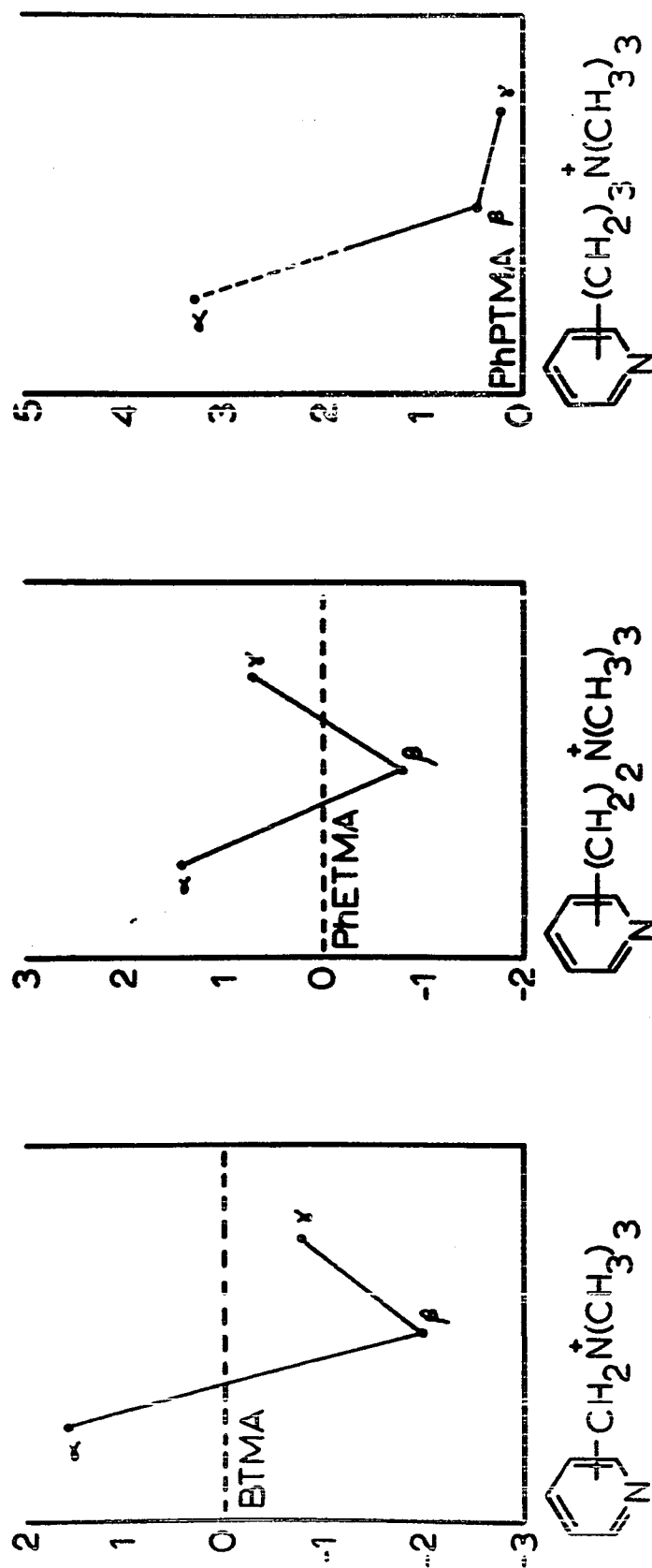
**Table 2**  
**Contracture of Frog Rectus Abdominis**  
**Pyridyl/Phenyl Comparison**

TABLE 2 - Contractures of Frog Rectus  
Pyridyl/Phenyl Comparison

| Compounds   | E.P.M.R. | $\pm$ | S.E.M. |
|---|----------|-------|--------|
| <u>x benzyltrimethylammonium<sup>-1</sup></u>       |          |       |        |
| alpha-pyridylmethyltrimethylammonium                | 37.7     | $\pm$ | 6.41   |
| beta-pyridylmethyltrimethylammonium                 | 0.010    | $\pm$ | 0.0011 |
| gamma-pyridylmethyltrimethylammonium                | 0.160    | $\pm$ | 0.0172 |
| <u>x phenylethyltrimethylammonium<sup>-1</sup></u>  |          |       |        |
| alpha-pyridylethyltrimethylammonium                 | 27.4     | $\pm$ | 1.73   |
| beta-pyridylethyltrimethylammonium                  | 0.063    | $\pm$ | 0.0069 |
| gamma-pyridylethyltrimethylammonium                 | 6.18     | $\pm$ | 0.402  |
| <u>x phenylpropyltrimethylammonium<sup>-1</sup></u> |          |       |        |
| alpha-pyridylpropyltrimethylammonium                | >2000    |       |        |
| beta-pyridylpropyltrimethylammonium                 | 3.02     | $\pm$ | 0.157  |
| gamma-pyridylpropyltrimethylammonium                | 1.77     | $\pm$ | 0.152  |

Figure 5  
 $\text{Log}_{10}$  Equipotent Molar Ratios  
Frog Rectus Abdominis  
Effect on Activity of Replacing the Pyridyl  
Group by a Phenyl Group  
(Recalculated Results)

FIG. 5       $\text{LOG}_{10}$  EPMR      FROG RECTUS  
 Effect on Activity of Replacing the Pyridyl Group by a Phenyl Group\*



\* Recalculated from Frog Rectus EPMR Results

Intermediate and statistically equivalent activities were demonstrated by both 1-phenylethyltrimethylammonium and 2-tetrahydrofurfuryltrimethylammonium which were approximately one-thirtieth as active as the reference drug. Statistical evaluations performed on the potency values of benzyltrimethylammonium and of the substituted analogue, 1-phenylethyltrimethylammonium, indicated equipotency ( $0.2 > P > 0.1$ ), but this is probably the result of the large standard error evident from bioassays with the latter compound. There was, however, a statistically significant difference between the potencies of 2-furfuryltrimethylammonium and its reduced analogue, 2-tetrahydrofurfuryltrimethylammonium ( $P < 0.05$ ).

In order to compare the effect on activity of replacing the pyridyl group by a phenyl group, these results have been recalculated and appear in figure 5 and table 2 using in each case the phenylalkyl derivatives as the standard reference compounds. Although no experiments were actually designed in this manner, the results have been co-ordinated to retain the standard errors. These results show that as far as the methyl series is concerned, the beta-pyridyl and gamma-pyridyl derivatives are more active than the phenylalkyltrimethylammonium, but the alpha-pyridyl compound is much less active. In the ethyl series the beta-pyridyl compound is the only one more active than

**Table 3**

**Maximum Contractures**

**Frog Rectus Abdominis**

**Pyridylalkyl and Phenylalkyltrimethylammonium Salts**

TABLE 3 - Maximum Contractures Frog Rectus Preparation

| Compounds                            | Relative Contractures* | ± | S.E.M. | n= |
|--------------------------------------|------------------------|---|--------|----|
| carbamylcholine (theoretical)        | 1.00                   |   |        |    |
| carbamylcholine (experimental)       | 0.99                   | ± | 0.04   | 3  |
| alpha-pyridylmethyltrimethylammonium | 0.70                   | ± | 0.04   | 4  |
| alpha-pyridylethyltrimethylammonium  | 0.81                   | ± | 0.03   | 5  |
| alpha-pyridylpropyltrimethylammonium | 0.91                   | ± | 0.02   | 3  |
| beta-pyridylmethyltrimethylammonium  | 0.92                   | ± | 0.03   | 4  |
| beta-pyridylethyltrimethylammonium   | 1.05                   | ± | 0.04   | 6  |
| beta-pyridylpropyltrimethylammonium  | 1.00                   | ± | 0.07   | 6  |
| gamma-pyridylmethyltrimethylammonium | 1.02                   | ± | 0.05   | 5  |
| gamma-pyridylethyltrimethylammonium  | 0.99                   | ± | 0.14   | 6  |
| gamma-pyridylpropyltrimethylammonium | 1.01                   | ± | 0.03   | 6  |
| benzyltrimethylammonium              | 0.85                   | ± | 0.04   | 5  |
| phenylethyltrimethylammonium         | 0.98                   | ± | 0.02   | 6  |
| phenylpropyltrimethylammonium        | 0.98                   | ± | 0.05   | 6  |

48

Expressed as a fraction of the theoretical contracture of carbamylcholine (1.00)  
n= number of determinations.



**Figure 6**  
**Frog Rectus Abdominis Contractures**  
**Pyridylalkyl- and Phenylalkyltrimethylammonium Salts**

**The shaded area represents the theoretical contracture  
(100%) of carbamylcholine (carbachol)**

**The bars indicate the standard errors of the means**



phenylethyltrimethylammonium. One rather interesting point resulting from this recalculation is the manner in which the alpha-pyridylmethyltrimethylammonium and alpha-pyridylethyltrimethylammonium salts, although much less active than their phenyl counterparts, become much closer in comparable activities. Phenylpropyltrimethylammonium was more active than all the pyridylpropyl analogues.

#### Frog Rectus Abdominis--Maximum Contractures

Further information was obtained by determining whether or not the test compounds could produce the maximum response attainable by the reference compound. The reference substance was carbamylcholine, which was initially assigned a theoretical value of 1.0 (100%). A value significantly less than this would indicate that the compound cannot for some reason mimic the contracture produced by the reference drug, and this would be indicative of a partial agonist. When the experiments were commenced it was noticed that the first maximum contractures appeared to be slightly greater than subsequent ones. For this reason carbamylcholine was tested against itself to provide a standard against which the test compounds could more fairly be judged.

The results of the experiments are shown in figures 6 and 6a and tables 3 and 3a and the majority of compounds appear to be as capable as the reference drug

**Table 3a**  
**Maximum Contractures**  
**Frog Rectus Abdominis**  
**Furfuryl and Benzyltrimethylammonium Analogues**

TABLE 3a - Maximum Contractures Frog Rectus Preparation

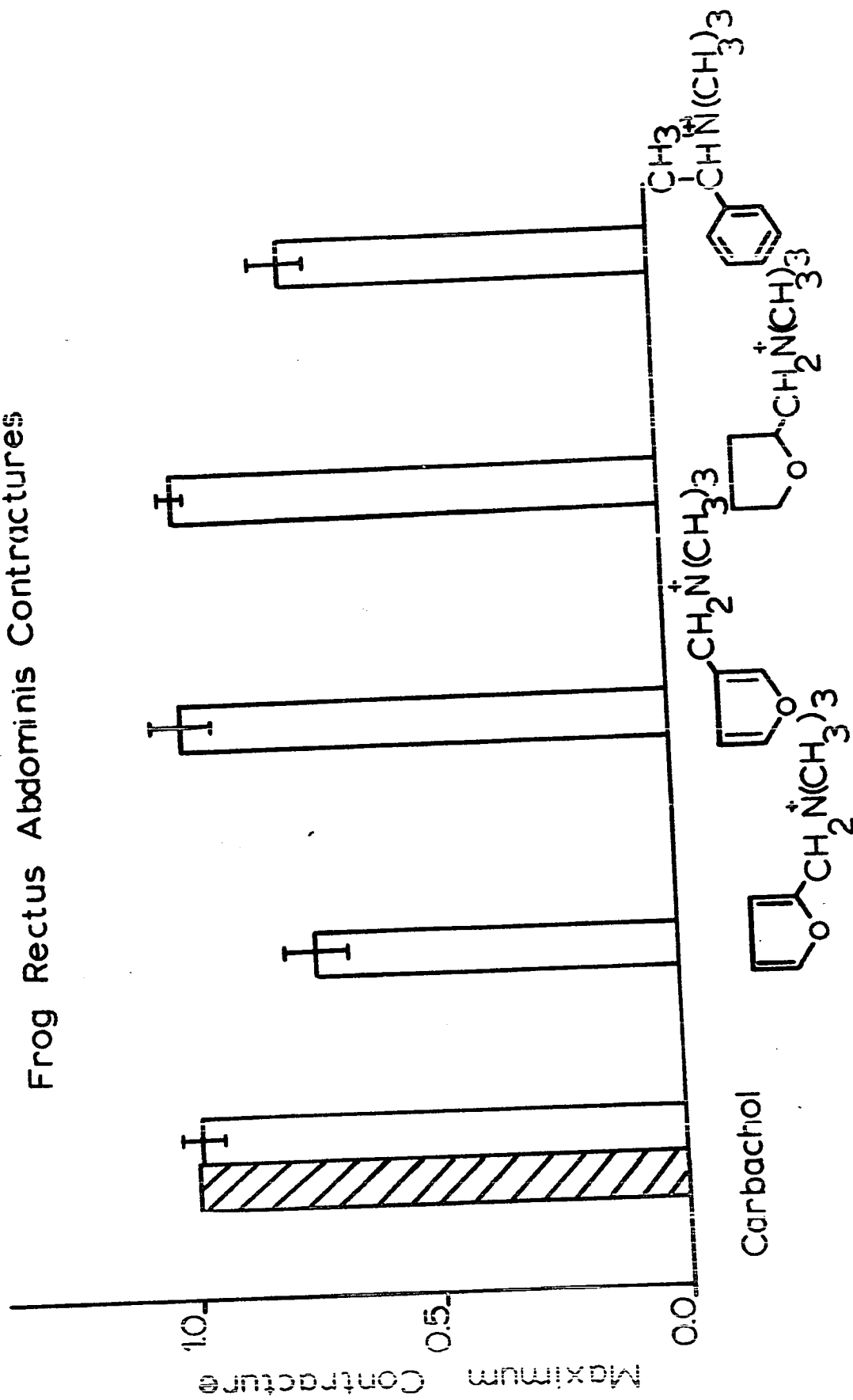
| Compounds                             | Relative Contracture* |      | S.E.M. |   | n= |
|---------------------------------------|-----------------------|------|--------|---|----|
|                                       | +                     | -    | +      | - |    |
| carbamylcholine (theoretical)         |                       | 1.00 |        |   |    |
| carbamylcholine (experimental)        | +                     | 0.99 | 0.04   |   | 3  |
| 2-furfuryltrimethylammonium           | +                     | 0.74 | 0.07   |   | 3  |
| 3-furfuryltrimethylammonium           | +                     | 1.00 | 0.06   |   | 3  |
| 2-tetrahydrofurfuryltrimethylammonium | +                     | 0.99 | 0.02   |   | 3  |
| 1-phenylethyltrimethylammonium        | +                     | 0.75 | 0.05   |   | 3  |

\* Expressed as a fraction of the theoretical contracture of carbachol (1.00)  
n= number of determinations.

Figure 6a  
Frog Rectus Abdominis Contractures  
Furfuryl and Benzyltrimethylammonium Analogues

The shaded area represents the theoretical contracture  
(100%) of carbamylcholine (carbachol)  
The bars indicate the standard errors of the means

FIG. 6a  
Frog Rectus Abdominis Contractures



at producing maximum responses. However, it should be noted that the alpha-pyridyl series as a whole showed significant inabilities to produce this response (alpha-pyridylmethyltrimethylammonium,  $P < 0.01$ ; alpha-pyridylethyltrimethylammonium,  $P < 0.01$ ; alpha-pyridylpropyltrimethylammonium,  $0.05 > 0.02$ ). It was found also that benzyltrimethylammonium appeared to be incapable of producing the maximal stimulus ( $P < 0.01$ ).

Two of the four compounds which were subsequently tested on this preparation were classifiable as partial agonists for they did not elicit full responses. They were 1-phenylethyltrimethylammonium ( $P < 0.01$ ) the substituted analogue of benzyltrimethylammonium and 2-furfuryltrimethylammonium ( $P < 0.01$ ). Interestingly, the isomer of this latter drug, 3-furfuryltrimethylammonium is a true agonist, as is the equivalently substituted reduced analogue, 2-tetrahydrofurfuryltrimethylammonium.

#### Rat phrenic nerve-diaphragm experiments

This preparation can be assumed to indicate the affinity of the test drug for the post-synaptic neuro-muscular receptor.

The effect of carbamylcholine was assigned a value of unity (1.00,  $\log_{10} 0.0$ ) and the test compounds were bioassayed against this substance. Values below



Table 4

Neuromuscular Blocking Effect  
Rat Phrenic Nerve-Diaphragm  
Pyridylalkyl and Phenylalkyltrimethylammonium Salts

TABLE 4. - Neuromuscular Blocking Effect-Rat Phrenic Nerve-Diaphragm

| Compounds                            | E.P.M.R. |   | S.E.M. |   | n= |
|--------------------------------------|----------|---|--------|---|----|
|                                      | +        | - | +      | - |    |
| gamma-pyridyltrimethylammonium       | 0.241    | + | 0.0049 | - | 3  |
| beta-pyridylethyltrimethylammonium   | 0.440    | + | 0.0407 | - | 5  |
| beta-pyridylmethyltrimethylammonium  | 0.538    | + | 0.017  | - | 3  |
| phenylpropyltrimethylammonium        | 0.643    | + | 0.0060 | - | 3  |
| beta-pyridylpropyltrimethylammonium  | 0.658    | + | 0.0281 | - | 4  |
| carbamylcholine                      | 1.00     |   |        |   |    |
| benzyltrimethylammonium              | 1.44     | + | 0.110  | - | 3  |
| phenylethyltrimethylammonium         | 1.51     | + | 0.109  | - | 3  |
| gamma-pyridylpropyltrimethylammonium | 1.69     | + | 0.050  | - | 3  |
| gamma-pyridylethyltrimethylammonium  | 10.4     | + | 0.901  | - | 4  |
| alpha-pyridylmethyltrimethylammonium | 41.6     | + | 2.95   | - | 2  |
| alpha-pyridylethyltrimethylammonium  | 50.5     | + | 10.00  | - | 3  |
| alpha-pyridylpropyltrimethylammonium | 52.7     | + | 9.28   | - | 3  |

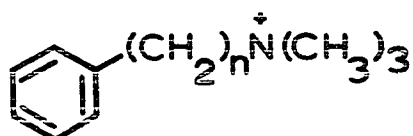
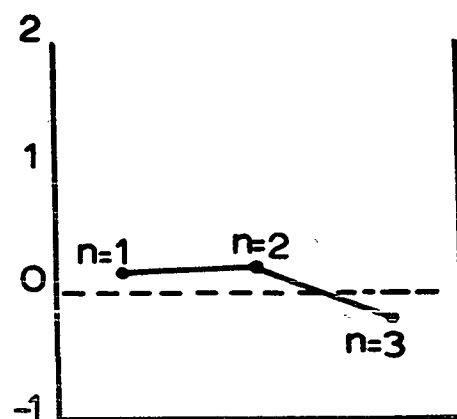
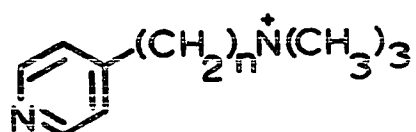
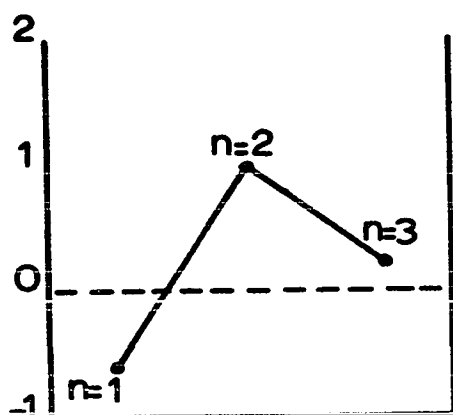
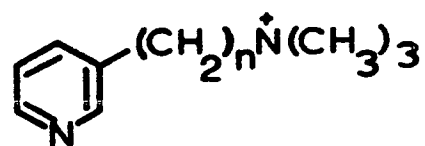
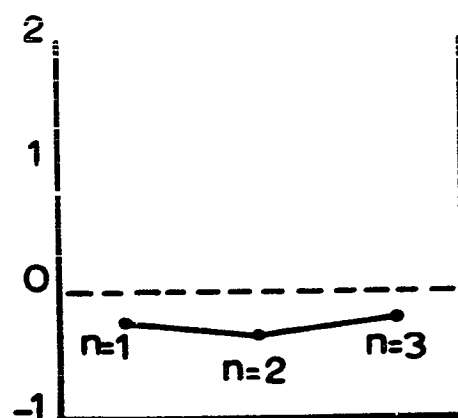
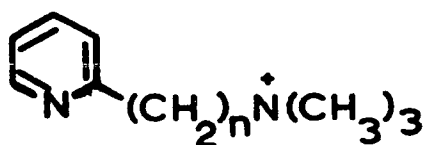
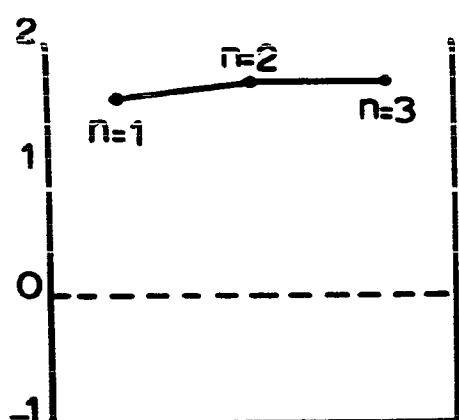
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Mean results arranged in order of decreasing potency.  
n= number of experiments.

Figure 7  
Rat Phrenic Nerve-Diaphragm  
 $\text{Log}_{10}$  Equipotent Molar Ratios  
Pyridylalkyl and Phenylalkyltrimethylammonium Salts

FIG. 7  
Rat Diaphragm

Log<sub>10</sub> Equipotent Molar Ratios



unity indicate a greater impedance to the action of liberated acetylcholine than that exerted by carbachol and values greater than unity demonstrate a lesser interference.

The results are contained in figure 7 (as  $\log_{10}$  E.P.M.R.s) and table 4. It may be seen that the beta-pyridylalkyltrimethylammonium salts were all significantly more effective than carbamylcholine in blocking the effect of liberated acetylcholine ( $P < 0.01$ ). However, the most effective compound was gamma-pyridylmethyammonium which demonstrated a great affinity for the acetylcholine receptor. The other compound that proved more effective than carbamylcholine was phenylpropyltrimethylammonium ( $P < 0.01$ ). There was no significant intra-series difference between the alpha-pyridylalkyltrimethylammonium compounds and all of these substances were much less active than carbamylcholine.

With the exception of gamma-pyridylethyltrimethylammonium which was about one-tenth as efficient as carbamylcholine, all the remaining substances proved to be slightly less "blocking" than the standard compound (benzyltrimethylammonium,  $0.1 > P > 0.05$ ; phenylethyltrimethylammonium,  $0.05 > P > 0.02$ ; gamma-pyridylpropyltrimethylammonium,  $P < 0.01$ ).

As described previously for the frog rectus

**Table 5**  
**Neuromuscular Blocking Effect**  
**Rat Phrenic Nerve-Diaphragm**  
**Pyridyl/Phenyl Comparison**

TABLE 5 - Neuromuscular Blocking Effect-Rat Phrenic Nerve-Diaphragm

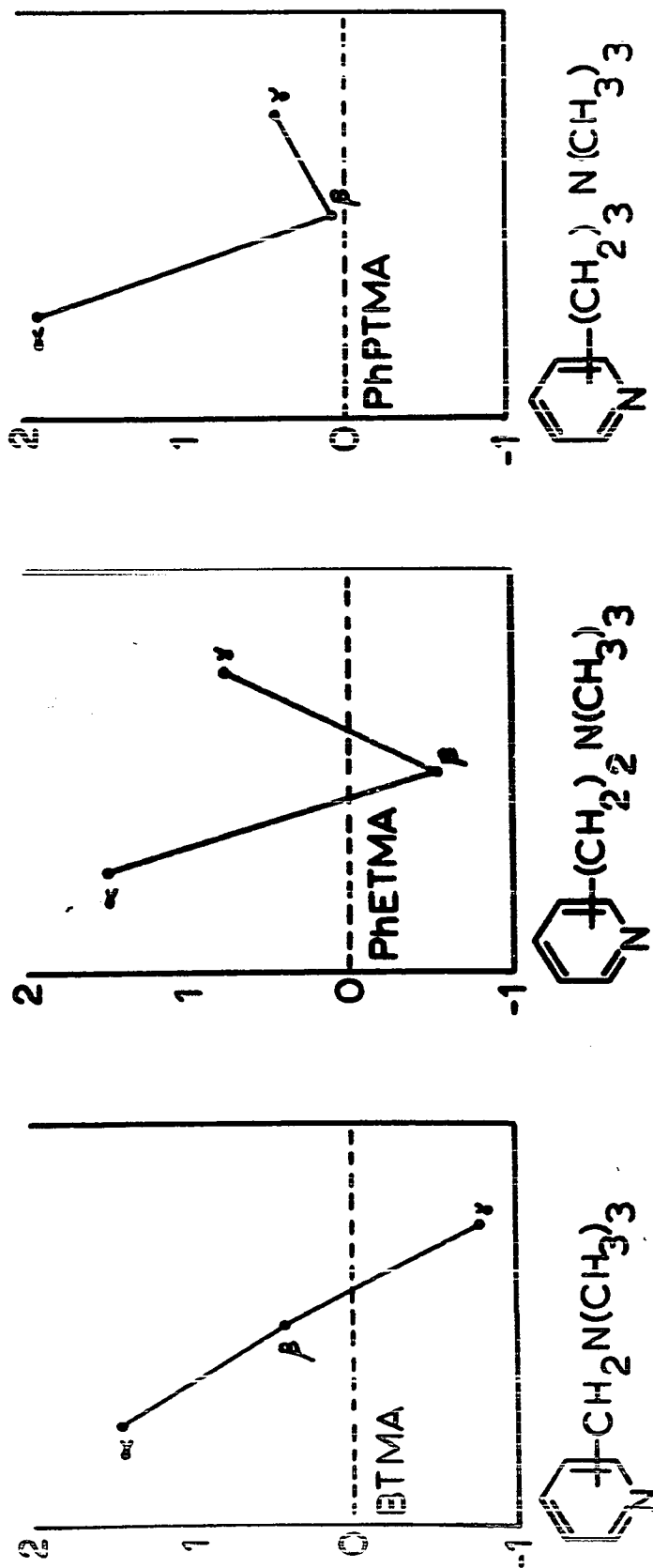
## Pyridyl/Phenyl Comparison

| Compounds   | E.P.M.R. | + | S.E.M. |
|---|----------|---|--------|
| <u>x benzyltrimethylammonium<sup>-1</sup></u>       |          |   |        |
| alpha-pyridylmethyltrimethylammonium                | 28.9     | + | 4.25   |
| beta-pyridylmethyltrimethylammonium                 | 0.373    | + | 0.0399 |
| gamma-pyridylmethyltrimethylammonium                | 0.167    | + | 0.0160 |
| <u>x phenylethyltrimethylammonium<sup>-1</sup></u>  |          |   |        |
| alpha-pyridylethyltrimethylammonium                 | 33.4     | + | 9.02   |
| beta-pyridylethyltrimethylammonium                  | 0.291    | + | 0.0477 |
| gamma-pyridylethyltrimethylammonium                 | 6.88     | + | 1.087  |
| <u>x phenylpropyltrimethylammonium<sup>-1</sup></u> |          |   |        |
| alpha-pyridylpropyltrimethylammonium                | 81.9     | + | 15.23  |
| beta-pyridylpropyltrimethylammonium                 | 1.02     | + | 0.053  |
| gamma-pyridylpropyltrimethylammonium                | 2.62     | + | 0.105  |

Figure 8  
 $\text{Log}_{10}$  Equipotent Molar Ratios  
Rat Phrenic Nerve-Diaphragm  
Effect on Activity of Replacing the Pyridyl  
Group by a Phenyl Group  
(Recalculated Results)



FIG 8  $\text{LOG}_{10}$  EPMR RAT DIAPHRAGM  
 Effect on Activity of Replacing the Pyridyl Group by a Phenyl Group



<sup>a</sup> Recalculated from Rat Diaphragm EPMR Results

**Table 4a**  
**Neuromuscular Blocking Effect**  
**Rat Phrenic Nerve-Diaphragm**  
**Furfuryl and Benzyltrimethylammonium Analogues**

TABLE 4a - Neuromuscular Blocking Effect-Rat Phrenic Nerve-Diaphragm

| Compounds                             | E.P.M.R. | ± | S.E.M. | n= |
|---------------------------------------|----------|---|--------|----|
| 1-phenylethyltrimethylammonium        | 1.07     | ± | 0.042  | 3  |
| 3-furfuryltrimethylammonium           | 2.28     | ± | 0.151  | 3  |
| 2-tetrahydrofurfuryltrimethylammonium | 2.80     | ± | 0.206  | 3  |
| 2-furfuryltrimethylammonium           | 6.05     | ± | 0.333  | 3  |

±\*

Mean result arranged in order of decreasing potency.  
n= number of experiments.

abdominis experiments, the effects of replacing the pyridyl group by a phenyl moiety were obtained by recalculating the results, each pyridylalkyl value being divided by the value obtained for the phenyl compound with the same side chain. The results are presented in table 5 and figure 8, the phenyl values being adjusted to unity.

It may be seen that the phenyl group shows a consistently higher affinity for the receptor than the alpha-pyridyl grouping. The beta- and gamma- pyridyl moieties however, may be appreciably more effective, or significantly less effective, than the phenyl group and these differences show intra-series variations. Thus, beta-pyridylmethyltrimethylammonium and beta-pyridylethyltrimethylammonium are about three times more effective than the equivalent phenyl compounds whereas beta-pyridylpropyltrimethylammonium and phenylpropyltrimethylammonium are equi-active. Gamma-pyridylmethyltrimethylammonium, the most effective "blocking" compound is more potent than benzyltrimethylammonium, but the other gamma- compounds are less effective than their phenyl analogues.

The results obtained from the determinations of the "blocking" abilities of the more recently obtained drugs are listed in table 4a. It can be seen that 1-phenylethyltrimethylammonium is equi-effective to carbamylcholine. This substance was also statistically

Table 6

Contractions of Guinea-Pig Ileum

(Treated with Hexamethonium)

Pyridylalkyl and Phenylalkyltrimethylammonium Salts

TABLE 6 - Contractions of Guinea-Pig Ileum (Treated with Hexamethonium)

| Compounds                            | E.P.M.R. | ± | S.E.M. | ** | n= |
|--------------------------------------|----------|---|--------|----|----|
| carbamylcholine                      | 1.00     |   |        |    |    |
| beta-pyridylmethyltrimethylammonium  | 15.96    | ± | 0.449  |    | 3  |
| alpha-pyridylmethyltrimethylammonium | 22.12    | ± | 0.235  |    | 3  |
| gamma-pyridylmethyltrimethylammonium | 31.76    | ± | 0.260  |    | 3  |
| benzyltrimethylammonium              | 39.03    | ± | 1.584  |    | 3  |
| beta-pyridylethyltrimethylammonium   | 438.8    | ± | 78.88  |    | 3  |
| phenylethyltrimethylammonium         | 439.9    | ± | 32.29  |    | 3  |
| gamma-pyridylpropyltrimethylammonium | 764.8    | ± | 23.85  |    | 3  |
| beta-pyridylpropyltrimethylammonium  | 2433     | ± | 170    |    | 2  |
| alpha-pyridylethyltrimethylammonium  | 3786     | ± | 370    |    | 3  |
| gamma-pyridylethyltrimethylammonium  | >20,000  |   |        |    | 2  |
| alpha-pyridylpropyltrimethylammonium | >20,000  |   |        |    | 2  |
| phenylpropyltrimethylammonium        | >20,000  |   |        |    | 3  |

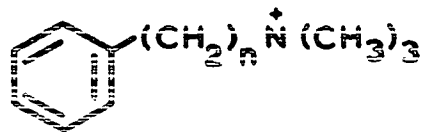
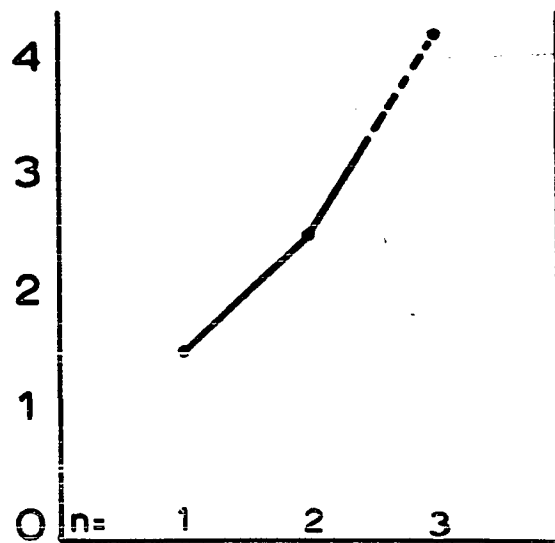
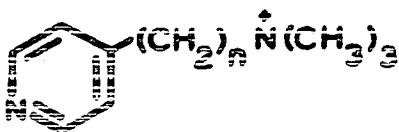
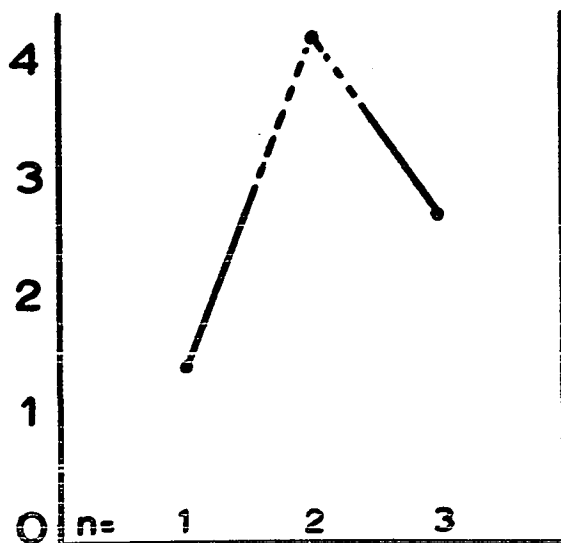
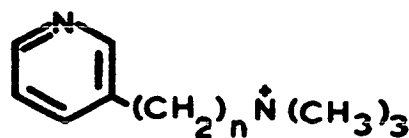
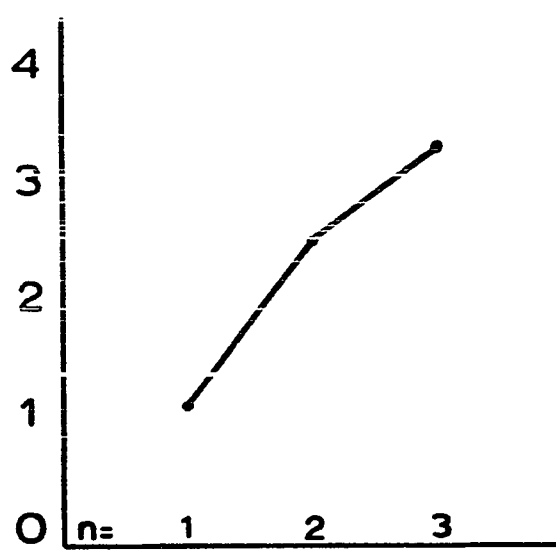
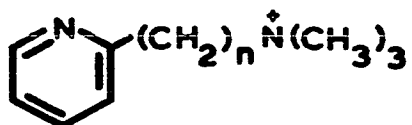
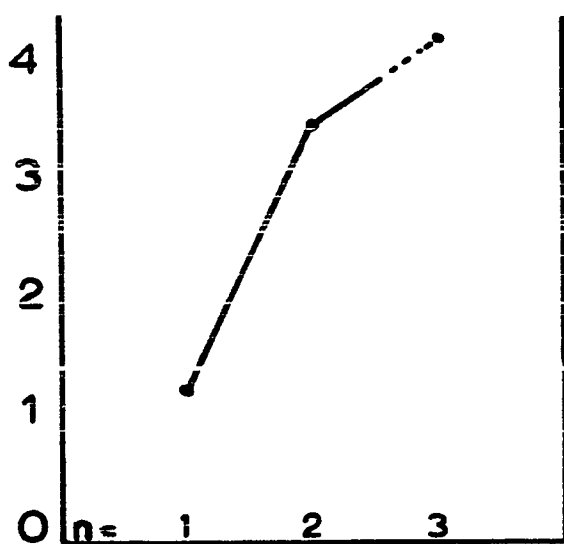
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Mean result arranged in order of decreasing potency.  
n= number of experiments.

Figure 9  
Guinea-Pig Ileum  
 $\text{Log}_{10}$  Equipotent Molar Ratios  
Pyridylalkyl and Phenylalkyltrimethylammonium Salts

FIG. 9 Guinea Pig Ileum

$\text{Log}_{10}$  Equipotent Molar Ratios





equipotent to the analogous benzyltrimethylammonium ( $0.1 > P > 0.05$ ), yet additional determinations might indicate a greater or lesser similarity.

The other drugs were less effective than the chosen standard. Activities between one-third to one-half of carbamylcholine were found for 3-furfuryltrimethylammonium and 2-tetrahydrofurfuryltrimethylammonium, whereas 2-furfuryltrimethylammonium was approximately one-sixth as effective a "blocker" as the standard drug.

#### Guinea-pig Ileum Experiments

The muscarinic activities of the homologous test compounds are shown in table 6 and the  $\log_{10}$  E.P.M.R.s denoted in figure 9. All the compounds proved to be less potent than the standard, carbamylcholine, and there were large intra-series decreases in activity as the side chains were extended beyond the methyl representatives. Beta-pyridylmethyltrimethylammonium was the most active of these initial test substances, being about one-sixteenth as active as carbamylcholine. This was followed by alpha-pyridylmethyltrimethylammonium, gamma-pyridylmethyltrimethylammonium and benzyltrimethylammonium. Beta-pyridylethyltrimethylammonium and phenylethyltrimethylammonium were of equal activity, roughly one-four-hundredth as active as carbamylcholine. Alpha-

Table 6a

Contractions of Guinea-Pig Ileum

(Treated with Hexamethonium)

Furfuryl and Benzyltrimethylammonium Analogues

TABLE 6a - Contractions of Guinea-Pig Ileum (Treated with Hexamethonium)

| Compounds                             | E.P.M.R. | ± | S.E.M. | n* |
|---------------------------------------|----------|---|--------|----|
| carbamylcholine                       | 1.00     |   |        |    |
| 2-furfuryltrimethylammonium           | 3.05     | ± | 0.218  | 5  |
| 2-tetrahydrofurfuryltrimethylammonium | 23.47    | ± | 0.650  | 3  |
| pilocarpine                           | 30.60    | ± | 1.122  | 3  |
| 3-furfuryltrimethylammonium           | 400.3    | ± | 12.84  | 4  |
| 1-phenylethyltrimethylammonium        | 4948     | ± | 245.3  | 3  |

\*n

Mean results arranged in order of decreasing potency.  
n= number of experiments.

pyridylethyltrimethylammonium was virtually inactive. The propyl homologues were even less active on the whole than the ethyl compounds. Gamma-pyridylpropyltrimethylammonium was the most active with an activity of less than one-seven-hundredth of that of carbamylcholine, and beta-pyridylpropyltrimethylammonium and phenylpropyltrimethylammonium were classified as inactive.

Table 6a illustrates the activities demonstrated by the additional compounds examined by this procedure. A comparative potency for pilocarpine was also determined and is included in this table.

It will be noted that 2-furfuryltrimethylammonium proved to be the most active of all the drugs investigated being about one-third as potent as carbamylcholine. The isomeric 3-furfuryltrimethylammonium compound was much less active and resembled beta-pyridylethyltrimethylammonium and phenylethyltrimethylammonium in potency. Although not as active as 2-furfuryltrimethylammonium, 2-tetrahydrofurfuryltrimethylammonium was one of the more potent agents being equiactive with alpha-pyridylmethyltrimethylammonium.

The remaining new drug, 1-phenylethyltrimethylammonium, was however, one of the least active compounds, only demonstrating one-five-thousandth of the potency of the standard agent.

Determinations on the potency of pilocarpine

Table 7  
 Contractions of Guinea-Pig Ileum  
 (Treated with Hexamethonium)  
 Pyridylmethyl/Phenylmethyl Comparison

TABLE 7 - Contractions of Guinea-Pig Ileum  
(Treated with Hexamethonium)

Pyridylmethyl/Phenylmethyl Comparison

| Compounds                               | E.P.M.R. | +<br>- | S.E.M. |
|---|----------|--------|--------|
| x benzyltrimethylammonium <sup>-1</sup> |          |        |        |
| alpha-pyridylmethyltrimethylammonium    | 0.567    | +<br>- | 0.0295 |
| beta-pyridylmethyltrimethylammonium     | 0.409    | +<br>- | 0.0232 |
| gamma-pyridylmethyltrimethylammonium    | 0.314    | +<br>- | 0.0407 |

against carbamylcholine revealed that the former clinically employed parasympathomimetic was approximately one-thirtieth as active as the chosen standard on this preparation. It was equipotent, therefore, with gamma-pyridylmethyltrimethylammonium ( $0.4 > P > 0.3$ ), but less active than 2-furfuryltrimethylammonium ( $P < 0.01$ ), alpha-pyridylmethyltrimethylammonium ( $P < 0.01$ ), and 2-tetrahydrofurfuryltrimethylammonium ( $P < 0.01$ ). Benzyltrimethylammonium was, however, less potent than pilocarpine ( $0.02 > P > 0.01$ ).

As the muscarinic activities of the homologous test substances declined so rapidly beyond the methyl derivatives, only these compounds were subjected to the pyridyl-phenyl comparison. The recalculated results are presented in table 7. It can be seen that all the pyridyl compounds are significantly more active than benzyltrimethylammonium ( $P < 0.01$  for all statistical determinations).

#### Unanaesthetised Chicks

The photographs taken to show the effects of the intra-peritoneally injected pyridylalkyltrimethylammonium and phenylalkyltrimethylammonium test compounds are exhibited in figure 10. As can be seen from these photographs all the compounds, except the alpha-pyridylalkyltrimethylammonium series, produced a prolonged

Figure 10

The Unanaesthetised Chick

Representative photographs taken to illustrate the effects of intra-peritoneal injections of the pyridylalkyl and phenylalkyltrimethylammonium compounds into young chicks.

alpha-PyMTMA -- alpha-pyridylmethyltrimethylammonium  
 alpha-PyETMA -- alpha-pyridylethyltrimethylammonium  
 alpha-PyPTMA -- alpha-pyridylpropyltrimethylammonium  
 beta-PyMTMA -- beta-pyridylmethyltrimethylammonium  
 beta-PyETMA -- beta-pyridylethyltrimethylammonium  
 beta-PyPTMA -- beta-pyridylpropyltrimethylammonium  
 gamma-PyMTMA -- gamma-pyridylmethyltrimethylammonium  
 gamma-PyETMA -- gamma-pyridylethyltrimethylammonium  
 gamma-PyPTMA -- gamma-pyridylpropyltrimethylammonium  
 BTMA -- benzyltrimethylammonium  
 PhETMA -- phenylethyltrimethylammonium  
 PhPTMA -- phenylpropyltrimethylammonium

Doses: 10mM dissolved in 1ml saline



# THE UNANAESTHETISED CHICK



CONTROL  
1ml. Saline I.P.



10µM alpha-PyMTMA



10µM beta-PyMTMA



10µM gamma-PyMTMA



10µM BTMA



10µM alpha-PyETMA



10µM beta-PyETMA



10µM gamma-PyETMA



10µM PhETMA



10µM alpha-PyPTMA



10µM beta-PyPTMA



10µM gamma-PyPTMA



10µM PhPTMA

µM=micromole

spastic paralysis with the head thrust back and legs extended (Buttle and Zaimis, 1949). Alpha-pyridylmethyltrimethylammonium elicited an extremely short-lived spastic paralysis prior to death. The other members of the alpha-series, alpha-pyridylethyltrimethylammonium and alpha-pyridylpropyltrimethylammonium, did not produce any observable change in the condition of the chicks at this dose level. In order to conserve materials, equipotent molar ratios and threshold levels were not determined on this preparation.

## VI. DISCUSSION

A. Some Considerations On  
Drug-Receptor Interactions

It is generally considered that a drug which acts on the 'receptors' of the effector cell enters into a complexing arrangement with these active sites. Furthermore, it is thought that this complex formation is, for drugs with efficacious characteristics, the first step in a process which ultimately affords the response. The response is presumably terminated by either the breakdown of the complex and the removal of the drug, with a subsequent return of the effector cell to its resting phase, or perhaps the response may be limited by some form of cellular fatigue with the persistence of the complex.

Most drugs dissociate from the receptor and are removed from the synaptic area by redistribution and/or catabolism, hence it is unlikely that the drug-receptor complex contains bonds with high energy properties. Therefore, the major types of bonds that are likely to participate are ionic bonds, hydrogen bonds and the weaker Van de Waal's forces. The ionic bonds are formed between suitably charged or partially charged groupings, and presumably there are both attracting and repelling

actions equivalent to 'unlike' and 'like' magnetic poles. The attracting force between the charged groupings varies with the square of the distance between the groupings and the maximum energy associated with this form of bond is of the order of 5 Kcal/mole. Hydrogen bonds have a similar maximum energy and are formed by a mutual-reciprocal sharing of protons by atoms such as nitrogen and oxygen which possess unshared electrons. Van de Waal's force arises from the time-variable distributions of electrons about positively charged atomic nuclei creating variable micro-dipoles which can attract one another. It is a weak force, of the order of 0.5 Kcal/mole (depending on the atoms concerned) and decreases exceedingly rapidly with increasing interatomic distance.

The interaction of drug and receptor presumably requires some stability to permit the subsequent reactions that yield the response to commence. At the same time, this stability must not be of such a magnitude as to be irreversible, if the tissue is to recover.

Stability is of course related to the energy associated with the bonds of the complex which, for dissociatability, should not exceed 10 Kcal/mole (Barlow, 1964). It would be pleasant indeed to know in greater detail the optimum energy requirements for the complex which initiates the stimulus, for then some theoretical perplexities might be resolved. Tetramethylammonium is an

agonist of acetylcholine (Clark and Raventos, 1937) and at the same time is a relatively unencumbered cation. In complexing with the receptor this ion could bond with an electrostatic force of some 5 Kcal/mole and therefore, this must represent a stability sufficient to result in a response. However, the potency of tetramethylammonium, relative to acetylcholine, is known to vary with regard to the site of agonistic action (see introductory section) and this is somewhat problematical. Although it is known that there are differences between the various types of cholinergic receptors, from the selective actions of several agonists and antagonists, the stability of the tetramethylammonium-receptor should be rather similar at each synaptic site. Practically however, for post-synaptic stimulation, higher concentrations of this cation are required at the 'muscarinic' synapse than at the ganglionic synapse (Clark and Raventos, 1937; Burn and Dale, 1915) and therefore there must be some impediment to 'muscarinic' stimulation by tetramethylammonium. Whether this is due to a differential permeability to the cation or a difference in the numbers of drug-receptor complexes required to bring diverse post-synaptic membranes to critical firing levels is a matter of speculation.

Other cations, for example acetylcholine and its congeners, are more efficacious than

tetramethylammonium. They possess other sites which may participate in bonding to receptors and thereby increase the stability of the adduct.

As has been indicated, the forces that are likely to play prominent roles in adduct stability are effective over a rather discrete range. Therefore there must be complementarity between the structure of the drug and the structure of the receptor and this implies a 'lock and key' relationship existing between the two. However it has recently been suggested (Triggle, 1965) that the receptor is not necessarily a rigid template for the drug, but rather that the drug can induce a conformational change in the receptor which is then more amenable to complex formation. Triggle has expanded this further and he has suggested that this structural change in the receptor may allow extracellular sodium ions into the cell initiating depolarisation. Although rather an attractive theory, the underlying response-producing mechanism is almost certainly a more complex affair than this suggests. Several other theories have been advanced to extend the drug-receptor complex to the final response (for example, Fatt and Katz, 1951; Del Castillo and Katz, 1957; McKay, 1963), but each suffers from the disadvantage that verification is a more difficult process than postulation.

Methylammonium cations are rather exclusive

groups in the study of structure and activity as they have an omnipresence in the drugs that are capable of depolarising the post-synaptic membrane. This suggests that they function as an essential triggering mechanism. Other molecules which have similar electronegative qualities, but which do not trigger the response, for example, the triethylammonium group, should be non-depolarising antagonists. Depolarising antagonists may be regarded as molecules that have the stimulating cation, but which form excessively stable complexes that do not rapidly dissociate and hence this limits repolarisation. These seem to be reasonable molecular explanations for the drugs with these pharmacological characteristics however, the state of 'partial agonism' is more difficult to rationalise. Examinations of the pharmacological activities of homologous series of drugs frequently show a gradual change from agonism, through partial agonism to antagonism. If, as has been argued above, agonists are substances which have a certain optimal persistence at the receptor (and the necessary triggering cation) and antagonists are viewed as compounds which create complexes which are more difficult to break down, then it may be presumed that partial agonists have complex stability properties which are intermediate. It is difficult to visualise how this concept may be extended to encompass the practical

evidence that partial agonists do not produce maximum stimuli. Although no theory of how the drug-receptor interaction initiates the response is without fault it is known that agonist-induced muscle contractions increase trans-membrane ionic movements (Born and Bülbring, 1956; Weiss, Coalson and Hurwitz, 1961; Durbin and Jenkinson, 1961; Bass, Hurwitz and Smith, 1964; Burgen and Spero, 1968), a situation resembling the ionic mobilisation during neuronal transmission (cf., Cole and Curtis, 1939; Hodgkin and Huxley, 1952). Of special interest to a discussion on partial agonism is the recent demonstration by Burgen and Spero (1968) that partial agonists produce a lesser mobilisation of ions than full agonists. Hence, partial agonists either limit the spread of excitation from the area of receptors, or alternatively, there is a distribution in responsiveness to spreading depolarisation in individual muscle cells. If indeed a degree of variability to the excitatory stimulus required for contractility occurs among the muscle fibres, then the partial agonist dilemma might be ameliorated.

If a certain total stimulus from the drug-receptor complexes is required to produce a maximal muscle contraction, this may be achieved by either a potent agonist (with high efficacy) interacting with a few receptors, or by a less potent agonist (with lower



efficacy) interacting with several receptors (Stephenson, 1956). A partial agonist, therefore, would be a compound of decidedly low potency that interacted with all available receptors, but did not produce the total stimulus. Therefore, a sub-maximal response would result from the contraction of only the more susceptible muscle cells.

Ariens (1966) has pointed out that some antagonists resemble equivalent agonists, whereas others, like atropine, he considers to be different. He proposes, therefore, that the receptor for atropine will not be identical with that of acetylcholine. Although it would appear to the author that there are outstanding similarities between the acetylcholine and atropine, and he would expect them to interact at the same receptor, Ariens's concept of two types of receptor is certainly worth consideration. The majority of the theories on drug-receptor interactions assume that the receptors are identical fractions of macromolecules, but this may not be so. It is possible that there is another population of receptors which, when complexed by a drug, reduce the sum stimulus. These secondary receptors may play a large role in partial agonism and equally they may be implicated when supra-maximal doses of full agonists produce, as they frequently do, a sub-maximal response. The existence of such inactivating

receptors would be difficult to verify in the normal manner, for by definition they produce inactivity, but as a concept they can explain away several of the irregularities to be found in synaptic pharmacology.

B. Consideration Of Structures And  
Synaptic Activities Of The  
Investigated Drugs

The bioassay techniques employed in these studies were such as to allow the determined activities to be divided into relative potencies at a nicotinic synapse and at a muscarinic synapse. For this reason, and because correlations between structure and activities appear to be unequal at these different sites of action, this discussion is divided under more appropriate sub-headings which denote the particular type of synapse and activity being considered.

Structure and Nicotinic Activity

The chemical characteristics of drugs which are capable of stimulating the post synaptic membrane of the neuromuscular junction have been briefly reviewed in an earlier section of this thesis. The apparent dependence on some form of cation was noted, as was the requirement for a further moiety (of variable structure) which would permit the total molecule to be transmuted to

acetylcholine. Attempts to suitably modify the nicotine molecule or the nicotinium ion have not, however, been accomplished in acceptable detail. Certainly the molecule can be easily related to benzyltrimethylammonium which was suitably aligned with acetylcholine by way of the simple alkyltrimethylammonium salts by Raventos (1937), but this explanation was considered too vapid and additionally the interesting toxicological studies of Craig (1934) seemed to refute this most direct analogy. While it was possible that these toxicological differences may have arisen through the multiple variables that exist in pharmacodynamics, such as absorption, metabolism, excretion, they may be based on a physiochemical diversity producing differing concentration of nicotinium ions. Nonetheless, the other earlier-mentioned works have supported a dissimilarity between the benzene ring and the beta-substituted position on the pyridine ring. It was decided to investigate how the other positions for mono substitution might compare, for these had not been extensively studied and the results obtained might contribute to a more thorough appreciation of nicotinic requirements.

The molecules that were initially prepared for this investigation included the pyridylalkyl-trimethylammonium salts mentioned previously which were assayed, with the equivalent phenyl series, by the

described techniques. Latterly some additional drugs were made and subjected to like activity assessments to improve the depth of the investigation and to further examine queries raised by the earlier researches.

The most obvious feature from the nicotinic experiments is the inconsistency of the intra-series activities with regards to the methylene groups in the side chain. Thus, on the frog rectus abdominis, beta-pyridylmethyltrimethylammonium is more active than the propyl analogue, whereas this situation is reversed in the gamma-pyridylalkyl and phenyl series. Moreover, the ethyl compounds do not necessarily demonstrate activities which are intermediate in character. There is an increase in activity for alpha-pyridylethyltrimethylammonium over alpha-pyridylmethyltrimethylammonium, a situation resembling the increasing depolarising ability of phenylethyltrimethylammonium over its methyl analogue. Beta-pyridylethyltrimethylammonium is, on the frog rectus abdominis, equiactive with the synthetic compound that most resembles nicotine, beta-pyridylmethyltrimethylammonium, whereas there is a most surprising decline in activity in extending the side chain in the gamma-pyridyl series from one carbon atom to two.

Additionally, there are differences in the overall activities of the different series. Thus, members of the alpha-pyridyl homologous series are

decidedly low in nicotinic potency and this is not essentially due to gross deviation from the nicotine molecule. The gamma-substituted pyridyl compounds, which also are less direct analogues of the tobacco alkaloid, have appreciable nicotinic activities, somewhat resembling the activities demonstrated by the phenylalkyl compounds. The beta-pyridyl homologues, with the same substitution as exists in nicotine, proved to be the most nicotinic series.

The recalculations performed to compare the effect of replacing any of the pyridyl moieties by the benzene ring ably demonstrate the inadvisability of regarding the two aromatic systems as interchangeable at all. It was concluded, therefore, that the pyridyl ring could not be regarded in any way as equivalent to an aliphatic chain of methylenes and that more subtle atomic interactions must be responsible for the variable depolarising activities expressed by the pyridyl substituted compounds. Before attempting a deeper analysis of this situation it would be pertinent to consider the achievements of other workers.

The arylmethyltrimethylammonium and some arylethyltrimethylammonium salts have been assayed before on postural skeletal muscles. Table 8 illustrates values obtained in these studies. Hamilton and Rubinstein (1968) compared the activities

Table 8  
Comparative Nicotinic Equipotent Molar Ratios

TABLE 8 - Comparative Nicotinic Equipotent Molar Ratios

|                                      | *    | 1c*    |
|--------------------------------------|------|--------|
| acetylcholine                        |      | 1.00   |
| carbamylcholine                      |      | 1.00   |
| nicotine                             | 1.00 | 1.28   |
| alpha-pyridylmethyltrimethylammonium | 55   | 1270.3 |
| alpha-pyridylethyltrimethylammonium  | 55   | 774.3  |
| beta-pyridylmethyltrimethylammonium  | 1.00 | 0.43   |
| beta-pyridylethyltrimethylammonium   | 0.28 | 0.217  |
| gamma-pyridylmethyltrimethylammonium | 1.00 | 3.28   |
| benzyltrimethylammonium              | 4.2  | 24.70  |
| phenylethyltrimethylammonium         | 8.7  | 3.46   |

\* Harlow and Hamilton (1962,a). Contracture of chick biventer cervicis.

\* Hamilton and Rubinstein (1968). Contracture of frog rectus abdominis.

(Rana pipiens).

of the pyridylmethyl- and phenylmethyltrimethylammonium drugs on the frog rectus abdominis preparation using acetylcholine as the standard compound. It will be noticed that there are most acceptable similarities between these results and those of the author considering the differing standards employed. (The similarities become highly relevant if one assumes acetylcholine to be roughly twice as potent as carbamylcholine. Chang and Gaddum in 1933 reported a somewhat larger potency difference which may however be the result of using rectus abdominis muscles from different frog species.) The table contains, in addition, results obtained by Barlow and Hamilton (1962a) on the chick biventer cervicis muscle. Although the reference compound in this investigation was nicotine it is obvious that there are differences between this preparation and the frog rectus abdominis preparation. Interestingly enough the alpha-pyridyltrimethylammonium compounds were still much less active than the analogous drugs. Barlow and Hamilton (1962a) also report the activities associated with pyridylmethyl- and pyridylethyl-dimethylammonium salts and an equivalent series of pyridylalkylpyrrolidine series. The dimethylamino compounds proved to be consistently less active than their quaternary trimethylamino analogues, thereby demonstrating the importance of charged onium



moeities. As the determined activities of the dimethylamino drugs were still much less than might have been expected, correlating the determined pKa's to concentrations of the quaternised ions, applied compounds must have been diluted in some way. This may have occurred by binding to non-functional membrane structures and/or the lipid soluble tertiary amines penetrated the cellular membrane, thereby reducing the extra-cellular, drug-receptor complexing concentrations.

The pyridylalkylpyrrolidines are of great interest because they contain the pyrrolidine ring as does the nicotine molecule. Barlow and Hamilton (1962a) examined the potencies of these substances in eliciting contracture of the chick biventer cervicis muscle. It was found that the beta-pyridylmethyl derivative was the most potent and the activity of the homologous ethyl compound was much lower. The alpha-pyridylmethyl- and the alpha-pyridylethylpyrrolidino compounds were amongst other drugs studied by Haglid and Wellings (1962a, b, c). These workers employed three biological assay techniques for examining the nicotinic activities of prepared drugs, comprising an isolated guinea-pig ileum preparation, the isolated rabbit jejunum preparation and the isolated rectus abdominis muscle of the frog *Rana temporaria*. It is regrettable that they do not describe their pharmacological methods in greater detail

for the untreated guinea-pig ileum preparation affords the nicotinic response indirectly, via stimulation of the plexal ganglia and there may also be sizeable muscarinic overtones contributing to the contractional response. Unfortunately the rabbit jejunum experiments are subjected to similar ambiguity for the amines may be liberating response-modifying catecholamines. In consequence, the frog rectus abdominis work alone serves as an indicator of nicotinic activity. When these results are compared to the above-mentioned determinations of Barlow and Hamilton, both groups used nicotine as the standard drug, some differences and similarities appear. Worthy of note are the low potencies of the alpha-pyridyl compounds on the frog rectus abdominis and the decline in activity as the alkyl group increases from the most active substance beta-pyridylmethylpyrrolidine to the ethyl homologue. In addition, this work of Haglid and Wellings reveals some interesting intra-series alterations in activity which compare with some of the author's observations. While it is admitted that the Haglid and Wellings compounds are more bulky, tertiary amines, and the experiments have been conducted on different species of the genus *Rana*, and moreover, that the orders of potency differ, the increasing activity in the alpha-pyridyl series and the decreasing activity in the gamma-pyridyl series are similar, as are the

relative magnitudes of these changes. The differences between the drugs and species limit the analysis of this situation, but it would appear that a degree of correlation exists between the experiments.

The above discussion has examined and compared the nicotinic activities of several pyridylalkylamines based on their abilities to contract striated muscle. However, this contractural response is of somewhat abbreviated use in an examination of structure-activity relationships for there is no indication of the affinity parameter. This defect was partially ameliorated by the experiments on the rat phrenic nerve-diaphragm preparation.

This exceedingly useful preparation serves as an indicator of relative affinity in the following way. The administered drug is considered to enter into complex formation with the post-synaptic receptors and thus interfere with the contractional response induced by electrically liberated quanta of acetylcholine. The depression observed may, however, arise by an interference into other synaptic events. For example, the drug could be acting pre-synaptically to reduce the synthesis or affect the release of the neuro-transmitter, or perhaps the drug has local anaesthetic properties which reduce the output of acetylcholine. Furthermore, the drug, in addition to

complexing with the receptor, could react with acetylcholinesterase and reduce its ability to inactivate the liberated neuro-transmitter, thereby presenting a misleading picture of the affinity of a drug for the receptor. In defense of the author's observations however, it is unlikely that these factors contribute to any appreciable extent. Firstly, drugs that inhibit the synthesis of acetylcholine are relatively gradual in onset and their effects are most noticeable during periods of rapid electrical stimulation when the turnover rate of acetylcholine is considerably increased. Secondly, quaternary ammonium salts are poor local anaesthetics because they do not penetrate the neural lipid layer, and thirdly, the effects of several of these substances on acetylcholinesterase have been examined (Barlow and Hamilton, 1962a) and these results would indicate a negligible participation by acetylcholinesterase inhibition in the experimental determinations.

The results from the experiments have been mentioned previously and the majority of these are readily relatable to the potencies described on the frog rectus abdominis. The alpha-pyridylalkyltrimethylammonium compounds are poor agonists on the latter preparation and the results from the rat diaphragm experiments indicate that this is due to a relatively

poor affinity for the receptor. Much greater affinity is demonstrated by the beta-pyridylalkyltrimethylammonium salts as might be expected from their potencies. However, the gamma-pyridyl homologous series behaves in a decidedly odd manner. The methyl derivative appears to have a most high affinity for the neuro-muscular junction receptor which declines rapidly as the alkyl chain increases to ethyl, and then return somewhat with the propyl homologue. These activities of the ethyl and propyl derivatives are not unexpected considering their abilities to depolarise the frog rectus, but gamma-pyridylmethyltrimethylammonium does appear to have, with only a moderate stimulating action, a profound affinity. However, the differences in potencies that are found when a compound is tested on various preparations, each purporting to demonstrate the same type of pharmacological action, probably render it unwise to too deeply attempt this correlation of efficacy and affinity, especially when these parameters are measured on preparations from different genera.

Some of the synthesised compounds have been prepared and tested on the rat diaphragm by Barlow and Hamilton (1962a) using nicotine as the standard drug. The values obtained therefore, differ quantitatively from those expressed in this thesis, where carbamylcholine was the chosen standard drug, but there is absolute

**Table 9**  
**Comparative Neuromuscular Blocking Effect**

TABLE 9 -- Comparative Neuromuscular Blocking Effect

|                                      | Barlow and                     |       |
|--------------------------------------|--------------------------------|-------|
|                                      | Hamilton (1962a) <sup>4*</sup> |       |
| alpha-pyridylmethyltrimethylammonium | 5.0                            | 41.6  |
| alpha-pyridylethyltrimethylammonium  | 5.0                            | 50.5  |
| beta-pyridylmethyltrimethylammonium  | 0.18                           | 0.538 |
| beta-pyridylethyltrimethylammonium   | 0.10                           | 0.440 |
| gamma-pyridylmethyltrimethylammonium | 0.063                          | 0.241 |

<sup>4\*</sup> Relative to a nicotine standard.

agreement in the relative order of the determined potencies (table 9).

The species dependent responses and the differing choices of standards render a critical evaluation of structure and activity somewhat difficult. Even so with few exceptions, there is sufficiently good agreement between the results of the author and those of others for the determined activities to be considered representative, although not easy to explain. Although superficially it would be true to conclude that the substances which can be easily allied to nicotine are exceedingly active and yet related compounds may possess this activity to a lesser degree, it would be a shallow and relatively uninformative conclusion. However, any deeper analysis requires an examination of the molecules in a more basic manner to determine what possible interactions may be effecting the varied activities.

The investigated pyridylalkyltrimethylammonium salts differ in two main ways. They possess side chains of from one to three methylene groups and the attachment of this chain to the pyridine ring occurs at the alpha-, beta-, or gamma- position. The wide disparities that came to light when the pyridyl-phenyl values were compared, demonstrated that the heterocyclic system is engaging in the overall activity picture by either



modifying the molecules prior to their entering into receptor binding, thereby presenting more or less acceptable intramolecularly bonded conformations, or alternatively the molecules are simply being presented to the receptor in the absence of such intramolecular alterations. Elaborations on biologically active conformers of the drugs could not be further pursued without considerations of what forces might be providing the intramolecular attractions or repulsions.

The pyridine ring system differs from the benzene ring in that it possesses a nitrogen atom of moderate electropositive characteristics. This atom is somewhat negatively charged (Longuet-Higgins and Coulson, 1947) and could attract by electrostatic forces, positively charged moieties. The cationic quaternary nitrogens extant in the studied drugs, are positively charged and might consequently be attracted to the heterocyclic atom. The strong possibility of such interactions occurring, especially where sterical conditions might allow reasonable approximations between the charges, prompted the measurements of such intra-nitrogen distances. Table 10 shows the values obtained from models constructed from Corey-Pauling blocks and three measurements were made on each molecular system. The minimum figures refer to the situations where the intra-atomic distances were at the positions

**Table 10**  
**Intra-Nitrogen Atomic Distances**

TABLE 10 - Intra-nitrogen Atomic Distances\* ( $\text{\AA}$ )

|                                      | Minimum | Maximum | Minimum-Planar |
|--------------------------------------|---------|---------|----------------|
| alpha-pyridylmethyltrimethylammonium | 2.7     | 3.6     | 3.5            |
| alpha-pyridylethyltrimethylammonium  | 3.3     | 4.7     | 4.3            |
| alpha-pyridylpropyltrimethylammonium | 3.0     | 4.9     | 3.6            |
| beta-pyridylmethyltrimethylammonium  | 4.2     | 4.7     | 4.4            |
| beta-pyridylethyltrimethylammonium   | 4.2     | 5.8     | 4.7            |
| beta-pyridylpropyltrimethylammonium  | 5.2     | 6.8     | 6.5            |
| gamma-pyridylmethyltrimethylammonium | 4.6     | 5.1     | 4.7            |
| gamma-pyridylethyltrimethylammonium  | 5.0     | 6.4     | 6.4            |
| gamma-pyridylpropyltrimethylammonium | 5.4     | 7.9     | 6.8            |

\* Measurements made from models comprised of Corey-Pauling units.

( $\text{\AA}$ ) = Angstrom Units.

of closest approach and the maximum values reflect measurements made at positions of maximum intra-nitrogen distances. The minimum-planar measurements were made in deference to the possibility of planarity being as important an issue in the receptor complexing of these drugs as it appears to be for the structurally similar phenylalkylamines (Clark, Dawes and Williams, 1968). The models were arranged, therefore, in the conformer which possessed maximum planarity co-incident with minimal intra-nitrogen distances.

These measurements reveal many interesting points worthy of further consideration. The high and equal levels of nicotinic activity possessed by the beta-pyridylmethyl and beta-pyridylethyl compounds may be reflected in both molecules having either minimum approach distances of about  $4.2\text{\AA}$  or common distances of  $4.7\text{\AA}$  which might be critical to optimal receptor reaction. As the  $4.7\text{\AA}$  distance recurs in gamma-pyridylmethyltrimethylammonium and alpha-pyridylmethyltrimethylammonium and these compounds are less active than the beta-pyridyl materials, the common  $4.2\text{\AA}$  would be favoured for maximum nicotinic potency. However, it must be remembered that the experiments with the rat phrenic nerve-diaphragm preparation revealed gamma-pyridylmethyltrimethylammonium to be a potent inhibitor to the action of electrically released pulses of acetylcholine and consequently the

intra-nitrogen distance of  $4.7\text{\AA}$  should not be too rapidly discarded. This of course brings the alpha series into focus for the above-mentioned alpha-pyridylethylamine also has this particular intra-molecular distance.

The alpha-pyridyl compounds as a whole was found to be the most exceptional series not because they were unexpectedly potent nicotinic agents, but conversely, because they were so poor. The model measurements show that the minimum figures for these compounds vary about a mean of  $3.0\text{\AA}$  and additionally, of the three positions for pyridyl substitution, the alpha position is the one where the attraction of the heterocyclic nitrogen for the cationic nitrogen engenders a minimum of steric interference from the general bulk of the ring system. Probably therefore, the preferred conformations are close to this minimal and these intra-nitrogen distances are unacceptable to the nicotinic receptor.

The molecules which have not been mentioned as yet do also possess reasonable nicotinic potencies, but do not present particularly striking intra-molecular distances. Nonetheless, the  $6.8\text{\AA}$  distance, common to beta- and gamma- pyridyltrimethylammonium, may indicate that these molecules can fit some form of nicotinic receptor which this distance is of importance, for these drugs are of similar potencies to carbamylcholine. (To what degree this latter receptor might be independent

from the former, is a matter of conjecture, but this is worthy of further study. Interestingly enough, a distance of approximately  $7\text{\AA}$  has been implicated at the ganglionic receptor (Gill and Ing, 1958), but as there are postulated charge differences this is probably serendipity.)

While the above discussion is virtually pure inference, it does relate well to the following points. Archer, Lands and Lewis (1962) have suggested that the conformer of acetylcholine in which the ester and the cationic nitrogen are in the 'cis' form about the ethylenic moiety is associated with nicotinic activity. The molecule of acetylcholine was constructed using Corey-Pauling units, the intention being to measure from the carbonyl oxygen to the cationic nitrogen under conditions which reflected as much as possible the proposed conformation. It was found that there were two prime conformations that might be considered as 'cis' arrangements. One has a carbonyl oxygen-nitrogen distance close to  $4.2\text{\AA}$  and the other is about  $4.7\text{\AA}$  between these atoms. This evidence demonstrates consistency between acetylcholine and the most active of the studied compounds and incidentally supports the hypothesis of Sekul and Holland (1961a) who, as stated previously, require a partial negative charge adjacent to the cation. It would appear, however, that this partial negative performs

a dual duty, not only entering into receptor bonding, but previously arranging the molecule into a more or less acceptable form. Additionally, the poor agonistic qualities of the alpha- series illustrates the spacial limitations that must be imposed on the mutual attraction which would support the modified theory proposed by Coleman, Hume and Holland (1965).

In 1955, Barlow and Dobson studied the activities of the N-methyl derivatives of nicotine. Whilst nicotine monomethiodide was a potent agonist there was equivalently less activity in the isomeric nicotine iso-methiodide and the dimethiodide. These latter molecules have the pyridine ring nitrogen methylated and hence cationic. Although this negligible potency may be due to repulsion by the receptor 'esteratic' site of the positive charge on the ring nitrogen, there would not be the intramolecular attraction between the two nitrogens mentioned above.

The additional compounds studied in the second part of this investigation proved to have interesting activities. Although initially prepared to examine muscarinic requirements, the furan-methyltrimethylammonium salts had remarkably similar general activities to the pyridyl analogues. Thus, 2-furfuryltrimethylammonium (cf., Ing, Kordik and Tudor Williams, 1952) was much less nicotinic than

**Table 10a**  
**Oxygen-Nitrogen Intra-Atomic Distances**



TABLE 10a - Oxygen-Nitrogen Intra-Atomic Distances<sup>a</sup> (Å)

|                                       | Minimum | Maximum | Minimum-Planar |
|---------------------------------------|---------|---------|----------------|
| 2-furfuryltrimethylammonium           | 2.6     | 3.9     | 2.9            |
| 3-furfuryltrimethylammonium           | 4.3     | 4.6     | 4.5            |
| 2-tetrahydrofurfuryltrimethylammonium | 2.8     | 3.9     | not applicable |

<sup>a</sup> Measurements made from models comprised of Corey-Pauling units.

(Å) = Angstrom Units.

3-furfuryltrimethylammonium, a situation which accords with the difference in activities associated with alpha- and beta-pyridylmethyltrimethylammonium. There would appear therefore to be some degree of interchangeability between pyridine and furan rings at the nicotinic receptor. Models of the furfurylamines were made and measurements taken from the oxygen to the cationic head nitrogen (table 10a). The oxygen atom of the furan ring would serve to attract a cation like the nitrogen of the pyridine ring, but probably less effectively. This may well be a reason why 3-furfuryltrimethylammonium is less active than beta-pyridylmethyltrimethylammonium, there being less pull to a critical  $4.2\text{\AA}$  distance. Similarly, 2-furfuryltrimethylammonium is more active than the analogous alpha-pyridyl compound and this again may reflect the less effective attractive force exerted on the quaternary nitrogen.

(±)-2-Tetrahydrofurfuryltrimethylammonium was, on the conducted bioassays, more nicotinic than its unsaturated derivative, 2-furfuryltrimethylammonium. Although the ring of the former compound is no longer planar (Beach, 1941) and the sample studied was a racemate (an equal mixture of stereo-isomers) the observed improvement in nicotinic activity over the latter (which might be higher still for one of the stereo-isomers) could be related to the relative

attracting powers of the heterocyclic oxygen atoms.

It is a feature of heterocyclic chemistry that furan rings are opened under milder acidic treatments than required to cleave the ether linkage present in a tetrahydrofuran system. As the first step in such an hydrolysis is considered to be protonation of the oxygen atom (Sykes, 1961) it is obvious that the oxygen of the furan is more electropositive than the oxygen of the tetrahydrofuran. In other words it would, under equivalent circumstances be more ready to attract a positively charged moiety. Thus, the higher nicotinic potency of the reduced furan ring compound is consistent with the lower degree of attraction between its oxygen and nitrogen atoms.

Although the majority of the compounds proved to be full agonists, there were some partial agonists detected. These comprised the alpha-pyridyl series, the furan analogue, 2-furfuryltrimethylammonium, and the compounds, benzyltrimethylammonium and 1-phenylethyltrimethylammonium. These drugs appeared to be incapable of maximally stimulating the frog rectus abdominis whatever concentration was employed.

The author's viewpoint on partial agonists has already been noted in the first part of the discussion and it would appear that the present findings demonstrate partial agonism to be a more complex problem than

previously considered. Whilst the alpha-pyridyl series and perhaps the 2-furfurylamine could be considered to be classical partial agonists, having the low efficacies and affinities which might not be expected to generate maximal stimuli, this does not fully extend to the other compounds. The major inconsistencies are that benzyltrimethylammonium and 1-phenylethyltrimethylammonium are partial agonists, even though they are as "potent" as 2-tetrahydrofurfuryltrimethylammonium (a full agonist) and are more effective at preventing acetylcholine quanta from stimulating the rat diaphragm than this latter drug. It would be necessary to study further the nature of this blockade of the diaphragm for each of these compounds for a more specific elucidation. (Alternatively, determinations of affinity as performed by Barlow, Scott and Stephenson (1967) would be a useful adjunct to this discussion.)

Although in the final account this behaviour may be traceable to the bonding energies of receptor-drug complexing (or perhaps to the postulated response-reducing receptors), there is no information presently available which could rationally explain the above-mentioned discrepancy. This part must, therefore, be considered unresolved.

### Structure and Muscarinic Activity

The compounds were assayed for muscarinic activity on the hexamethonium-treated guinea-pig ileum as described previously. It was found that the pyridylmethyltrimethylammonium compounds and benzyltrimethylammonium were approximately twenty to forty times less potent than carbamylcholine and the activities demonstrated by their homologues showed that extension of the side chain by more than one methylene moiety was extremely detrimental to muscarine-like potency.

The reason for investigating muscarinic characteristics in these nicotine analogues followed from the communication by Hamilton and Rubinstein (1968) who, using acetylcholine as a standard drug, found comparative activities for these agents at producing saliva from the decentralised submaxillary gland of the cat and stimulating the muscarinic component of the superior cervical ganglion of this animal. The latter results from these studies are presented, along with those of the author in table 11. Quantitatively, the results appear to be of a completely different order of magnitude, but this could reflect the lability of acetylcholine. This is strongly supported by the high potency described for pilocarpine which appears from "in vitro" assays to be much less active than acetylcholine (Van Rossum,

Table 11  
Comparative Muscarinic Equipotent Molar Ratios

TABLE 11 - Comparative Muscarinic Equipotent Molar Ratios\*\*

|                                      | Hamilton and       |               |
|--------------------------------------|--------------------|---------------|
|                                      | Rubinstein (1968)* |               |
| acetylcholine                        | 1.00               |               |
| carbamylcholine                      |                    | 1.00          |
| alpha-pyridylmethyltrimethylammonium | 0.05 ± 0.010       | 22.12 ± 0.235 |
| beta-pyridylmethyltrimethylammonium  | 0.05 ± 0.002       | 15.96 ± 0.449 |
| gamma-pyridylmethyltrimethylammonium | 0.12 ± 0.021       | 31.76 ± 0.260 |
| benzyltrimethylammonium              | 0.17 ± 0.033       | 39.03 ± 1.584 |
| pilocarpine                          | 0.05 ± 0.007       | 30.65 ± 1.122 |

\*†† Results give E.P.M.R. ± S.E.M.

†† Contraction of the nictitating membrane of the cat.

Cornelissen, de Groot, and Hurkmans, 1960). The assay of pilocarpine against carbamylcholine performed on the treated guinea-pig ileum proved pilocarpine to be of a similar potency to the aryltrimethylammonium compounds. This evidence was considered to indicate that acetylcholine was being extensively hydrolysed by esterases in the "in vivo" cat experiments and that the more recently determined muscarinic responses are more truly representative of intrinsic potency.

The most interesting feature of the muscarinic activities elicited by the compounds that were initially tested, is the potency of the arylmethyltrimethylammonium compounds and the relative inactivity of the longer-chain members. This provides further examples for the Cavallito and Gray hypothesis (1960) which associates muscarinic activity with a protuberant "methylene neck". Nonetheless, although further examples are provided for the hypothesis, the full inter-relationship between the muscarinic activity of these compounds and acetylcholine is not more obvious.

It was mentioned, when nicotinic activity was correlated to the structures of the tested drugs that beta-pyridylethyltrimethylammonium could approximate the intra-nitrogen distances of beta-pyridylmethyltrimethylammonium which in turn can be associated with 'cis'-like conformers of acetylcholine. However, the



ethyl analogue is not excessively muscarinic, whereas the methyl derivative is quite active. Therefore, muscarinic characteristics are not dependent on these intra-nitrogen distances. Indeed, this independence is confirmed by the relatively similar potencies of the other pyridylmethyltrimethylammonium compounds and benzyltrimethylammonium, which has no electropositive ring nitrogen. Although no experiments were designed to give a direct comparison, the most active pyridyl compound beta-pyridylmethyltrimethylammonium is apparently a little more than twice as potent as benzyltrimethylammonium.

There is a similarity between alpha-pyridylmethyltrimethylammonium and 2-furfuryltrimethylammonium, the "muscarinic" agent investigated by Fellows and Livingston (1940). These substances differ in heterocyclic moieties, but both have the hetero-atom in the same relative position to the cationic head. It is not too surprising, therefore, that alpha-pyridylmethyltrimethylammonium is muscarinic for it could be assumed to interact at similar active sites to the furfurylamine derivative. However, the other arylmethyltrimethylammonium compounds could not be so accommodated unless the furan ring could be considered to be somewhat equivalent to the pyridine ring. It was postulated that if this was so then the isomeric 3-furfuryltrimethylammonium should have a muscarinic

potency that might be allied to the activities of the other "methylene neck" substances. In addition, if this compound possessed this activity, the convenient structural relationship between muscarine and acetylcholine, referred to in an earlier section, would require re-examination.

This compound was synthesised and bioassayed against carbamylcholine. It was found to have a relative muscarinic potency that associated well with the lower activity, longer chain homologues of the pyridyl and phenyl series. It supported, by default, the relationship between acetylcholine, muscarine and 2-furfurylamine, but did not uphold an inter-relationship between the unsaturated cyclic moieties, furan, benzene, pyridine.

(+)-2-Tetrahydrofurfuryltrimethylammonium was prepared and assayed to see to what extent the saturation of the furan ring affected muscarine-like activity. It was found, as Fellows and Livingston (1940) previously described, that the reduced ring reduced potency to about one-tenth. Nonetheless, the sample assayed was a racemate which renders it likely that one of the stereo-isomers might be somewhat more active.

The 2-substituted furfurylamines can be related to muscarine and so, to some extent, can alpha-pyridyl-methyltrimethylammonium. The remaining arylmethyammonium

derivates cannot be included in this relationship and alternative analogies must be examined.

The arylmethyltrimethylammonium compounds may be being considered by the receptor as simply equivalent to an alkyltrimethylammonium, all the ring structures being reduced to a  $C_{3-4}$  chain. (If this is so it is surprising that the electropositive nitrogen atom is so passive in this regard.) Alternatively, these compounds could be regarded as members of the group of unsaturated muscarine-like agents examined by Jacob, Marszak, Bardisa, Marszak-Fleury and Epstein (1952), the ring unsaturation replacing unsaturation in alkyl chains. Possibly the receptor for such compounds has an area of unsaturation which can provide an active site for bonding to unsaturation in a drug molecule. Hence, these compounds would fit the receptor in a different manner to acetylcholine, sharing an anionic active site, but differing in secondary complexings.

Clearly this matter is not yet terminated and additional studies are essential. Such studies would require a wider examination of the importance of unsaturation (or cyclopropanyl moieties) (cf., Armstrong, Cannon and Long, 1968) to muscarine-like activity, and might usefully employ partially reduced derivatives of the presently studied arylmethyltrimethylammonium compounds. A further study which would be of interest

would be the effect of methyl substitution on these compounds. This might lead to a resolving of the duality posed by alpha-pyridylmethyltrimethylammonium which, with a methyl on the other carbon atom adjacent to the hetero-atom might show a many-fold increase in activity over the parent compound (cf., Ing, Kordik and Tudor Williams, 1952).

### Structure and Synaptic Selectivity

These studies have again demonstrated, from the dissimilarities of the drug-induced responses, that differences must exist between the muscarinic and nicotinic receptors. In addition, some structural features have been illuminated which seem to confer relative specificity of the site of cholinergic activity. These may be usefully considered when future compounds are designed for therapeutic effectiveness with a minimum of contra-lateral colinergic side-effects.

Muscarinic specificity is apparent from the decline of activity with alkyl chain extension. Even so, arylmethyltrimethylammonium compounds can possess appreciable nicotinic activities which would make them less useful for any selective actions. However, if the compound additionally possessed the "methylene neck" cation in a similar arrangement to that adopted in alpha-pyridylmethyltrimethylammonium and 2-furfuryl-

trimethylammonium, undesirable nicotinic characteristics should be largely abated. It was considered earlier that this arrangement may induce conformational changes which are not acceptable to the nicotinic receptor. If this is indeed true, this contribution to muscarinic specificity arises by discrimination at the nicotinic site.

2-Furfurylmethyltrimethylammonium has been used clinically as a parasympathomimetic and the experiments on the ileum show it to be a very potent drug. However, it also possesses some degree of nicotine-like activity. If these present serious cholinergic side-effects (which is probably unlikely considering the differences between experimental dosage levels--see Appendix II), alpha-pyridylmethyltrimethylammonium (after the required pre-clinical evaluations) might prove to be an acceptable alternative which may have a better muscarinic/nicotinic ratio.

In the main, compounds that show nicotinic specificity seem to do so by having depressed abilities to stimulate the more selective muscarinic receptors. Nonetheless, this very fact can be used constructively to prepare compounds showing a high level of synaptic selectivity. From the completed studies it would be expected that almost exclusive nicotinic activity would be demonstrated by drugs with long side chains (that do not support muscarine-like actions) and in which distances

of  $4.2\text{\AA}$ ,  $4.7\text{\AA}$ , or  $6.8\text{\AA}$  can be approximated between a positively charged methyl-substituted nitrogen and an electropositive atom. These conditions are not complete, however, for nicotinic selectivity is present in a substance which is an arylmethyl derivative. This substance is nicotine and the nicotinic and muscarinic qualities of its simpler analogue, beta-pyridylmethyltrimethylammonium, readily demonstrates that the pyrrolidine ring of the tobacco alkaloid participates in its classical selectivity. Although this indicates support for the earlier-mentioned comment of Haglid and co-workers (Erdtman, Haglid, Wellings and von Euler, 1963a), that, "the intact pyrrolidine ring is a definite contributing factor to the biological activity of nicotine", this may be an excessively cautious conclusion. Perhaps only the substitution on the methylene carbon atom (which is an intrinsic part of the pyrrolidine ring) is required to differentiate receptors and hence confer synaptic specificity. This would then correlate to the increased specificity demonstrated by acetyl-alpha-methylcholine. Substituted beta-pyridylmethyltrimethylammonium compounds have not as yet been prepared to confirm this theory, but there is some evidence to support it.

Using benzyltrimethylammonium as a model for the above pyridylmethyltrimethylammonium, the substituted

analogue, 1-phenylethyltrimethylammonium was prepared and examined for muscarinic and nicotine-like potency.

This compound proved to be equi-nicotinic to its progenitor, yet its muscarine-like activity was profoundly reduced. As this drug appears to be equi-potent on the rat diaphragm, this substitution must exert its selectivity-conferring properties by making it less able to meet muscarinic requirements, rather than dramatically increasing its affinity for the nicotinic receptor.

However, as dissimilarity has been demonstrated between the benzene ring and pyridine ring the final analogy for the nicotinic selectivity of the classical drug itself must depend on the studied synaptic properties of 1-(3-pyridyl)-ethyltrimethylammonium.

Finally, the results from the "in vivo" experiments on the chicks corroborate the "in vitro" experiments. All of the pyridyl and phenylalkyl-trimethylammonium compounds with moderate to high nicotinic potencies produced the expected spasticities.

## VII. SUMMARY AND CONCLUSIONS

This thesis examines the abilities of the isomeric pyridylalkyltrimethylammonium and phenylalkyltrimethylammonium salts (with alkyl chains varying from one to three methylenes) to mimic synaptic behaviour that is associated with the classical drugs nicotine and muscarine. Four additional compounds were introduced into this study. They were analogous furan derivatives and a further arylalkyltrimethylammonium. Although some of these substances have been examined using similar bioassay techniques by other workers, this study re-assesses the quantitative activities against the stable parasympathomimetic, carbamylcholine, and further, it includes compounds not previously evaluated.

On conclusion of the determinations attempts were made to correlate the calculated relative potencies to the chemical structures of the drugs.

The pharmacological researches and results are summarised as follows:

1. Nicotine-like characteristics were quantitatively investigated on the frog rectus abdominis in vitro



and the rat phrenic nerve=diaphragm in vitro.

Qualitative experiments on the skeletal muscles of the chick demonstrated depolarising activities for the pyridyl and phenyl series.

2. Muscarine-like characteristics were shown on the guinea-pig ileum in vitro with interference from nicotinic interactions prevented by hexamethonium.
3. Inconsistencies between the determined potencies of the phenyl and pyridyl substituted drugs showed that the pyridine ring was not contributing to nicotinic activity in the same way as the phenyl system.
4. The decline in nicotinic potency on changing pyridyl substitution from beta- to alpha- was similarly detected in the analogous 3-furfuryltrimethylammonium and 2-furfuryltrimethylammonium compounds.
5. With regards to nicotine-like characteristics, the beta-pyridyl homologous series was most active, lesser activity was found for the gamma- series and the alpha- compounds were of very low potencies. Activities similar to those of the gamma-pyridyl compounds were noted for the phenylalkylamines.
6. A theory was advanced to explain the low nicotinic potencies of the alpha-pyridylalkyltrimethylammonium compound and analogous 2-furfuryl derivatives. It assumed that intra-molecular, partial ionic,

attractive forces might stabilise conformers which would then not be acceptable to the nicotinic receptor.

7. The higher activities demonstrated by the other compounds were related to intra-atomic distances of 4.2Å, 4.7Å and 6.8Å between hetero-atoms of the unsaturated ring systems and the cationic head. The first two distances were found to coincide with the carbonyl oxygen to cationic nitrogen in two 'cis'-like conformers of acetylcholine.
8. Benzyltrimethylammonium, 1-phenylethyltrimethylammonium, 2-furfuryltrimethylammonium and all the alpha-pyridyl compounds did not maximally stimulate the frog rectus abdominis. It would appear, therefore, that they are partial agonists.
9. Examinations of muscarinic characteristic revealed that high potencies (greater or equivalent to pilocarpine) were associated with the arylmethyltrimethylammonium drugs and extension of the alkyl side chain cause a profound decline in this activity.
10. Interpretations of structure-activity relationships did not follow those considered for nicotinic qualities and alternatives were considered. It was suggested that all the six-membered rings

might, as suggested by previous researchers, be judged as equivalent to a butyl or propyl chain or, alternatively, there may be some association between these compounds and others in which unsaturation promotes muscarinic qualities.

11. Structural arrangements were considered which appeared to yield specificity to the cholinergic site of action. It was considered that these might be employed in future drug syntheses where activity at a particular synapse would be a desirable feature.
12. The benzyltrimethylammonium analogue, 1-phenylethyltrimethylammonium, was tested for nicotine-like and muscarine-like characteristics. It was found to be similarly nicotinic, yet much less muscarinic, to benzyltrimethylammonium. This provides a further example of the specificity conferred on a molecule by alkyl substitution and in turn supports the author's theory that like substitution is largely responsible for the classical stimulant actions of nicotine.

In common with most researches in this field which attempts the tenuous path between structure and activity there are more questions raised than answered. In this regard many new studies have been suggested in

the text and it is probable that most rewarding conclusions await their successful completion. However, even this present study would have been improved by more refined indications of affinity parameters at both nicotinic and muscarinic sites. In addition, the use of hexamethonium in the guinea-pig ileum experiments renders these somewhat poly-pharmacological, for subtle interactions, quite apart from the desired ganglion blocking effect, may be playing a less than innocent role. Future researches on muscarine-like qualities would, therefore, better employ in vitro preparations which are without ganglionic components.

The rationale for using acetylcholine in vivo studies does not require justification, yet its lability must be borne in mind when it is employed in determinations of relative potency. It is suggested that better indications of true potency would be obtained in these experiments if a stable parasympathomimetic was also included in these studies.

APPENDIX I

# APPENDIX I

## Formulae for the physiological organ bathing solutions.

|   | Clarke's | Krebs'                              | Tyrodé's |
|---|----------|-------------------------------------|----------|
| Sodium Chloride                           | 65g      | 69g                                 | 80g      |
| Sodium Bicarbonate                        | 2g       | 21g                                 | 10g      |
| Glucose                                   | 20g      | 20g                                 | 10g      |
| Potassium Chloride (10% w/v)              | 14ml     | 35ml                                | 20ml     |
| Calcium Chloride (Molar)                  | 10.8ml   | 25.6ml                              | 18ml     |
| Sodium Dihydrogen Phosphate (5% w/v)      | 2ml      | -                                   | 10ml     |
| Potassium Dihydrogen Phosphate (5% w/v)   | -        | 34.4ml                              | -        |
| Magnesium Sulphate Heptahydrate (10% w/v) | -        | 14.6ml                              | 26ml     |
| Aerating gas                              | air      | O <sub>2</sub> + 5% CO <sub>2</sub> | air      |

APPENDIX II

### Legend to Appendix II

| <u>Cmpd.</u> | <u>Compound</u>                             |
|--------------|---|
| 1            | - carbamylcholine                           |
| ii           | - alpha-pyridylmethyltrimethylammonium      |
| iii          | - beta-pyridylmethyltrimethylammonium       |
| iv           | - gamma-pyridylmethyltrimethylammonium      |
| v            | - alpha-pyridylethyltrimethylammonium       |
| vi           | - beta-pyridylethyltrimethylammonium        |
| vii          | - gamma-pyridylethyltrimethylammonium       |
| viii         | - alpha-pyridylpropyltrimethylammonium      |
| ix           | - beta-pyridylpropyltrimethylammonium       |
| x            | - gamma-pyridylpropyltrimethylammonium      |
| xi           | - benzyltrimethylammonium                   |
| xii          | - phenylethyltrimethylammonium              |
| xiii         | - phenylpropyltrimethylammonium             |
| xiv          | - 2-furfuryltrimethylammonium               |
| xv           | - 3-furfuryltrimethylammonium               |
| xvi          | - 2-tetrahydrofurfuryltrimethylammonium     |
| xvii         | - 1-phenylethyltrimethylammonium            |
| xviii        | - pilocarpine                               |
| F.R.A.*      | - Frog Rectus Abdominis                     |
| R.P.D.*      | - Rat Phrenic Nerve-Diaphragm               |
| G.P.I.*      | - Guinea-Pig Ileum (Hexamethonium Treated)  |
| M            | - Molarity (of applied solutions)           |
| $\mu$ M      | - micromoles (contained in 50ml organ bath) |



## APPENDIX II

### Maximum and Minimum Doses/Solutions of Drugs Employed in Quantitative Potency Determinations

| <u>Cmpd.</u> | <u>F.R.A.*</u>  | <u>R.P.D.*</u>                        | <u>G.P.I.*</u>  |
|--------------|---|---------------------------------------|---|
|              | <u>Min:Max</u>  | <u>Min:Max</u>                        | <u>Min:Max</u>  |
| i            | $5 \times 10^{-6} \text{M} : 2 \times 10^{-5} \text{M}$   | $1.5 \mu\text{M} : 3 \mu\text{M}$     | $5 \times 10^{-8} \text{M} : 2 \times 10^{-7} \text{M}$   |
| ii           | $5 \times 10^{-3} \text{M} : 1 \times 10^{-2} \text{M}$   | $60 \mu\text{M} : 120 \mu\text{M}$    | $2 \times 10^{-6} \text{M} : 4 \times 10^{-6} \text{M}$   |
| iii          | $1 \times 10^{-6} \text{M} : 5 \times 10^{-6} \text{M}$   | $0.75 \mu\text{M} : 1.5 \mu\text{M}$  | $2 \times 10^{-6} \text{M} : 4 \times 10^{-6} \text{M}$   |
| iv           | $2 \times 10^{-5} \text{M} : 4 \times 10^{-5} \text{M}$   | $0.30 \mu\text{M} : 0.60 \mu\text{M}$ | $3 \times 10^{-6} \text{M} : 6 \times 10^{-6} \text{M}$   |
| v            | $5 \times 10^{-4} \text{M} : 1 \times 10^{-3} \text{M}$   | $75 \mu\text{M} : 150 \mu\text{M}$    | $4 \times 10^{-4} \text{M} : 8 \times 10^{-4} \text{M}$   |
| vi           | $1 \times 10^{-6} \text{M} : 5 \times 10^{-6} \text{M}$   | $0.75 \mu\text{M} : 1.5 \mu\text{M}$  | $5 \times 10^{-5} \text{M} : 1 \times 10^{-4} \text{M}$   |
| vii          | $1 \times 10^{-4} \text{M} : 2 \times 10^{-4} \text{M}$   | $15 \mu\text{M} : 30 \mu\text{M}$     | -- : $5 \times 10^{-3} \text{M}$                          |
| viii         | -- : $1 \times 10^{-2} \text{M}$                          | $75 \mu\text{M} : 150 \mu\text{M}$    | -- : $5 \times 10^{-3} \text{M}$                          |
| ix           | $2 \times 10^{-5} \text{M} : 4 \times 10^{-5} \text{M}$   | $1 \mu\text{M} : 2 \mu\text{M}$       | $2.5 \times 10^{-4} \text{M} : 5 \times 10^{-4} \text{M}$ |
| x            | $1 \times 10^{-5} \text{M} : 2 \times 10^{-5} \text{M}$   | $2.5 \mu\text{M} : 5 \mu\text{M}$     | $7 \times 10^{-5} \text{M} : 1.4 \times 10^{-4} \text{M}$ |
| xi           | $1 \times 10^{-4} \text{M} : 4 \times 10^{-4} \text{M}$   | $2.5 \mu\text{M} : 5 \mu\text{M}$     | $4 \times 10^{-6} \text{M} : 8 \times 10^{-6} \text{M}$   |
| xii          | $2 \times 10^{-5} \text{M} : 4 \times 10^{-5} \text{M}$   | $2.5 \mu\text{M} : 5 \mu\text{M}$     | $5 \times 10^{-5} \text{M} : 1 \times 10^{-4} \text{M}$   |
| xiii         | $1 \times 10^{-5} \text{M} : 2 \times 10^{-5} \text{M}$   | $1 \mu\text{M} : 2 \mu\text{M}$       | -- : $5 \times 10^{-3} \text{M}$                          |
| xiv          | $5 \times 10^{-4} \text{M} : 1 \times 10^{-3} \text{M}$   | $10 \mu\text{M} : 20 \mu\text{M}$     | $3 \times 10^{-7} \text{M} : 6 \times 10^{-7} \text{M}$   |
| xv           | $5 \times 10^{-5} \text{M} : 1 \times 10^{-4} \text{M}$   | $4 \mu\text{M} : 8 \mu\text{M}$       | $4 \times 10^{-5} \text{M} : 8 \times 10^{-5} \text{M}$   |
| xvi          | $2.5 \times 10^{-4} \text{M} : 5 \times 10^{-4} \text{M}$ | $4 \mu\text{M} : 8 \mu\text{M}$       | $2.5 \times 10^{-6} \text{M} : 5 \times 10^{-6} \text{M}$ |
| xvii         | $2.5 \times 10^{-4} \text{M} : 5 \times 10^{-4} \text{M}$ | $1.5 \mu\text{M} : 3 \mu\text{M}$     | $5 \times 10^{-4} \text{M} : 1 \times 10^{-3} \text{M}$   |
| xviii        | -- : --   | -- : --                               | $3 \times 10^{-6} \text{M} : 6 \times 10^{-6} \text{M}$   |

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